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Relevance of vermicomposting in present era in organic waste valorization and bioremediation: An urgent need in environmental protection

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ABSTRACT

The environment friendly and economically affordable technology for the treatment of biodegradable solid waste is vermicomposting. Earthworms are used in the vermicomposting process to turn biodegradable organic waste into vermicast, which resembles humus. The end result of the vermicomposting process, which involves the cooperation of earthworms and bacteria, is vermicompost. The ecology is seriously threatened by the rate at which solid garbage is being produced around the world. If adequate precautions are not implemented, biodegradable contaminants will bring about an unpleasant odour and unclean situation. By minimising the negative consequences of garbage, this technique turns the waste into valuable manure. Vermicompost is a top-notch organic fertiliser that helps in organic farming and also has pest-repelling qualities. In the present paper production of vermicompost, role of earthworm in preparing composting and use of vermicompost in heavy metal removing from water bodies, chelating of contaminants in soil, influence in plant growth and development and pest and disease prevention abilities have been highlighted.

1. Introduction

Increased population rate and rapid urbanization leads to a proportionate increment in global waste generation which is estimated to be 1.3 billion metric tons approximately at present and the amount is expected to get doubled by 2025 (Samal *et al*., 2019). Of the total solid waste generated majority are of organic in origin. Therefore, management of wastes is a global concern and the management system is directly proportional to environmental and socio-economic factors, that's why majority of the developing countries are unable to avail updated technologies for safe disposal of waste due to economical barrier. The mostly used waste management technologies are composting, landfilling, combustion, waste to energy conversion, etc and some of them adversely affects environment. Landfill dumping leads to ground water contamination and release of higher amount of greenhouse gases. Carbon dioxide and other dangerous gases are released in large quantities during the combustion process. Besides use of sewage sludge as fertilizer on agricultural land again release some toxic chemicals to the plants and thus causes soil contamination and also affects activities of useful soil microbes. Therefore, the most beneficial methods of solid waste management are recycling, and reuse. For this, one of the practical and environmentally benign method for turning commercial and household trash into high-quality compost is vermicomposting. Earthworms are added to organic waste during the vermicomposting process, when they progressively transform the garbage into vermicompost, which is a humus-like substance which is black in colour and odourless. Vermiculture refers to the large production of earthworms in garbage, while vermicomposting refers to the use of earthworm technology to valuable waste management (Bhat *et al*., 2018). Besides vermicompost are preferred to be the soil medicine and is utilised extensively for crop production. It enhances plant

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productivity, soil health, and the possibility for disease resistance. Numerous reports back up the idea that adding vermicompost to soil could boost the yield of a variety of crops and plants (Biswas *et al*., 2021; Biswas *et al*., 2022).

2. Role of earthworm in vermicomposting

Earthworms and gut microbes work together during vermicomposting to break down the organic waste into tiny bits. By using their muscles, earthworms break down bigger organic particles into smaller ones in the first stage, and then the gut bacteria break down the newly generated smaller particles in the second step. The microbial breakdown process is accelerated by the smaller particles' greater surface area, which is employed as a microbe attachment site. The biodegradable waste is broken down by microorganisms in the second stage. Carbon dioxide and water are the last by-products of aerobic decomposition. The variety of bacteria that break down trash and the material's biodegradability are key factors in the waste degradation process. The rate of decomposition increases if the waste materials are readily biodegradable. The organic matter is digested by earthworms and builds up inside of them. The composting bed becomes more porous as a result of earthworm burrowing activity, allowing ambient oxygen to seep into the filter bed. In composting beds, aerobic conditions predominate and promote the growth of aerobic microorganisms. Water and nutrients disperse everywhere on the bed because of its porous texture. Earthworms secrete mucus and coelomic fluid, which combine with organic materials and start the degrading process when it is damp. Additionally, earthworms function as a buffering agent and maintain a pH of 7 in the composting bed. Earthworms alter the makeup of trash, reducing organic carbon content and the C:N ratio while preserving macro- and micronutrients (Bhat *et al*., 2018). Because it reduces the toxicity of the three wastes, earthworm bioconversion of industrial wastes and sludges is beneficial (Bhat *et al*., 2018). Heavy metals in industrial sludges can be detoxified by chloragocyte cells and the bacteria that live in earthworm guts (Srivastava *et al*., 2005). The various types of earthworms applied for waste recycling and vermicomposting have been presented in Table 1.

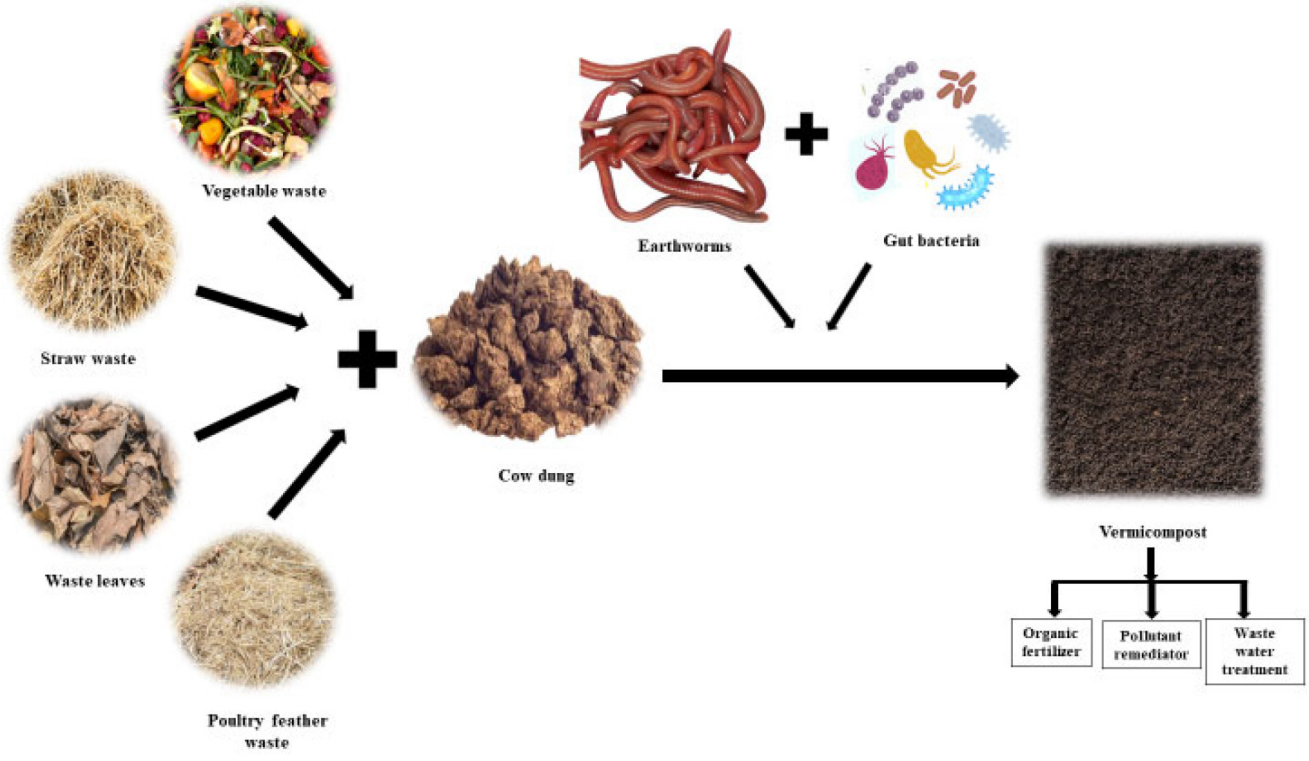

Figure 1: Scheme of vermicomposting.

Table 1

Biological criteria to classify earthworms used for waste management.

Earthworm category	Representatives	Characteristic features
Anecic	*Lumbricus terrestris*, *L. friendi*, *L. polyphemus*	They are of large size having light pigmentation with moderate reproduction rate and long life cycle. They generally live inside deep soil layer and are thus deep burrower, however, are not much efficient in waste recycling. They are phytogeophagous and for feeding they use litter and soil andexcrete organo-mineral faeces.
Endogeic	*Aporrectodea caliginosa*, *A. rosea*, *Octolasion cyaneum*, *Allolobophora chlorotica*	They are of medium size having low or no pigmentation with low reproduction rate and medium life cycle. They live mostly in upper layer of soil and burrow up to 10-30 cm representing medium burrowing capacity. Some species engaged in vermicomposting. They are geophagous and for feeding they use organic matter of soil and excrete organo-mineral faeces.
Epigeic	*Eisenia fetida*, *Lumbricus rubellus*, *Perionyx excavatus*, *Eudrilus eugeniae*	They are of small size, highly pigmented with high reproduction rate and short life cycle. They are most efficient species for vermicomposting. They preferably live between 3-10 cm having very much low burrowing capacity. They are phytophagous and for feeding they use leaf litter and animal excreta and excrete holorganic faeces.

By using *Eisenia fetida* as a vermicomposting agent, it is possible to significantly alter the bacterial composition and diversity while also reducing the toxicity and overall concentration of heavy metals. Different digestive enzymes, such as cellulase, amylase, phosphatase, protease, mannase, and lipase, are released by microbes and the gut of earthworms to aid in the breakdown of different organic matter components, such as starch, lignin, cellulose, and hemicellulose. Earthworms release proteins and a variety of nitrogenous compounds, which enhance the nitrogen content in the compost bed. In the compost bed, the nitrogenous molecules that are released mineralize and become available to the plants. By burrowing, casting, grazing, and dispersing earthworms alter the biochemical and physical characteristics of trash (Samal *et al.,* 2019). The vermicomposting method involves two stages: (1) During the active phase, earthworm digestion alters the physical and microbiological properties of waste materials. (2) During the maturation phase, earthworms leave the compost's matured layer and go to the new, undigested layers. The density of earthworms, species, pace of trash consumption, and method all affect how long the active phase lasts. Due to a variety of gut-related activities, the physical and biological features of organic waste in the earthworm stomach changed. The earthworm consumes partially decomposed organic waste, eliminates harmful microorganisms, adds helpful gut microbes, and ultimately pushes the digested materials to the surface of the soil as vermicast. The dynamics of the microbial population entirely alter in this manner. Old bacterial species that were present in the materials which the vermicomposting process consumed vanishe, while new bacterial species proliferated.

Some of the bacterial species associated with vermicomposting are *Aeromonas hydrophila*, *A. caviae*, *Sphingobacterium* sp., *Azospira* sp., *Bacillus subtilis*, *Flavobacterium* sp., *Fluviicola* sp., *Myroides* sp. and others. By secreting enzymes, *Aeromonas hydrophila* accelerates the breakdown of organic material. Protein hydrolysis is carried out by Saprospiraceae, which also utilises amino acids as a source of carbon and energy. In the process of the anaerobic food chain, proteins are broken down to CH_4 and CO_2. *Sphingobacterium* sp. has been discovered to breakdown steroidal oestrogens and aromatic chemicals like methylbenzene, pyrene, and phenanthrene. Fluviicola aid in the digestion of sludge. Delta-proteobacteria have been found to break down other microorganisms (such as bacteria and yeast) by secreting a variety of hydrolytic enzymes, including amylase, lipase, proteases and enzymes that break down cell walls. *B. subtilis* create a range of enzymes that

can break down a range of natural substrates, and induce the creation of beneficial biofilms. *Aeromonas caviae* have the ability to create biofilms, break down organic materials, and convert nitrate to nitrite. Flavobacterium N_2O should be released by improving the denitrification process. Myroides species producing emulsifying agents will aid in the biodegradation of proteins (Samal *et al.,* 2019).

3. Waste management and bioremediation efficacy of vermicompost

3.1. Heavy metal removal from water bodies

Vermicompost is found to associated with the release of heavy metals. Several reports support the fact. For the total removal of Cd^{2+}, Cu^{2+}, chromium (as $Cr_2O_7^{2-}$), Ni^{2+}, and Zn^{2+} from synthetic aqueous solutions and galvanoplastic effluents, Jordao *et al.,* (2002) packed glass columns with sufficient amounts of vermicompost and observed the satisfying results. When Matos and Arruda (2003) used vermicompost to treat chemical laboratory effluents, they were able to successfully adsorb Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+}. Vermicompost was utilised by Jordao *et al.* (2010) to remove Fe^{2+} and Al^{3+} from artificial aqueous solutions as well as from industrial wastewaters coming from a mineral processing facility. Another explanation for the prevalence of unspecific adsorption can be found in the parallels between the charge densities for Zn^{2+} and Cu^{2+} as well as Pb^{2+} and Cd^{2+}. This is in addition to the significant excess of negative charges in the vermicompost structure. When Kaushik and Garg (2003) used *Eisenia fetida* for the composting of textile sludge, they found that the resultant vermicompost had 25% less Cr and 11.5-38.2% less Zn. When employing cow dung as a bulking agent, Gupta *et al.* (2007) found a considerable reduction in Pb^{2+}, Cd^{2+}, and Cu^{2+} in the final vermicompost produced from water hyacinth. Domnguez-Crespo *et al.* (2012) discovered a dramatic reduction in Ni and Zn in the final vermicompost after composting sewage sludge using *Eisenia fetida*.

3.2. Removal of contaminants from soil

Vermicompost have been used to successfully lower the amount of a herbicide called 3-(3,4- dichlorophenyl)-1,1-dimethylurea, or diuron, that is present in soils (Fernandez-Bayo *et al.,* 2008). As a result of diuron's ($C_9H_{10}C_{l2}N_{2O}$) acceptable polarity and high affinity for the hydrophilic groups of vermicompost, this compound is distributed throughout several strata of modified soils. Similar to this, Fernandez-Bayo *et al.,* (2007) investigated the impact of vermicompost on imidacloprid ($C_9H_{10}CIN_5O_2$) pesticide mobility in numerous Spanish soils. Vermicompost was accountable for the considerable retention of imidacloprid, as seen for diuron, which was expected from polarity considerations (Fernandez-Bayo *et al.,* 2008).

3.3. Use of vermicompost for plant growth and productivity

Vermicompost is suitable for plant growth because it is rich in microbial floraincluding bacteria, fungi and actinomycetes. Additionally, vermicompost contains hormones and enzymes that promote plant growth and inhibit plant diseases. Numerous researchers have noted that the final vermicompost made from cow dung, paper mill sludge, and sewage contains significant amounts of humic chemicals, which are crucial for plant development and growth (Bhat *et al.,* 2018). Figure 2 represents the nutritional content of vermicompost as explained by Biswas *et al.* (2022). After establishing a symbiotic interaction between fungus and the roots of sorghum, Gutierrez-Miceli *et al.* (2008) observed a considerable boost in plant development when fertilizers produced from leachates of vermicompost containing sufficient amount of potassium, nitrate, and phosphate were administered to sorghum cultures. Biswas *et al.* (2021) applied CFPH (Chicken feather protein hydrolysate) amended vermicompost for yield improvement of tomato plants. Biswas *et al.* (2022) applied vermicompost in combination of egg shell dust for cultivation of Capsicum and observed satisfying results. Zhao *et al.* (2017) used chicken manure vermicompost for cultivation of cucumber (*C. sativus*) on sandy loam soil and observed higher fruit yield and quality under continuous cropping conditions. Similarly, vermicompost produced with combination of rice waste, rice husk ash and coconut fiber when applied for tomato (*Lycopersicon* sp.), the yield was higher compared to the controls and other treatments (Truong *et al.,* 2017). The NPK uptake, plant height, leaf area, shoot weight, eardiameter, weight of the husked and unhusked ears and weight of the husked ear per plot all significantly increased with VC application for sweet corn (*Zea mays*) production. The impact increased as VC concentration increased (Muktamar *et al.,* 2017). Similarly, when Lemon grass (*Cymbopogan flexuosus*) was grown with VC made from cow dung, plant waste, and lemongrass waste, the plants' height, tillernumber, herb yield, and oil content all rose (Sasikala *et al.,* 2016). The ability of VC made from industrial cassava waste to affect maize (*Zea mays*) development and as soil conditioners to improve salt-affected soils was evaluated. In comparison to the control, VC displayed higher plant height and more total dry matter of maize (Oo *et al.,* 2015). *Phaseolus vulgaris*, a bean plant, was grown with a mixture of cow manure, wheat straw and melon waste amended with VC. VC increased plant height, number of pods, leaf area, grain dry weight, length, volume, dry weight of roots, pod dry weight, number of grains, dry weight of biomass, and nodule number (Gardezi *et al.,* 2013). Comparative research was done to see how well tomato (*Lycopersicon esculentum*), bell pepper (*Capsicum anuum*)

and strawberry (*Fragaria* spp.) cultivation responded to VC made from food and recycled paper waste, cow manure and inorganic fertiliser. When compared to inorganic fertiliser, VC increased the growth and productivity of each of the species under study (Arancon *et al.,* 2003).

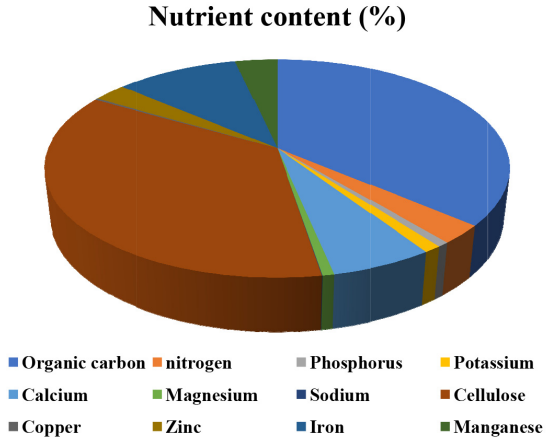

Figure 2: Nutritional profiling of vermicomposting (Biswas *et al*., 2022).

3.4. Role of vermicompost on pests and disease control

Plant disease is efficiently controlled by adding organic amendments to soils with low levels of organic matter and microbial activity (Pathma and Sakthivel, 2012). Thermophilic compost has been shown to have disease-suppressing capabilities against a variety of phytopathogens, including *Rhizoctonia*, *Plasmidiophora brassicae*, *Phytopthora*, *Gaeumannomyces graminis*, and *Fusarium*. As organic supplements increase the microbial population and variety, microbial conflict may be one of the potential causes of disease suppression. Traditional thermophilic composts only support a limited number of microbes, whereas non-thermophilic vermicomposts are abundant sources of microbial diversity and harbour a considerable number of antagonistic bacteria, acting as efficient biocontrol agents and assisting in the eradication of diseases brought on by soil-borne phytopathogenic fungi (Pathma and Sakthivel, 2012) Insect pests, plant parasitic nematodes and a wide variety of microbiological illnesses can all be suppressed by VC, according to numerous research. Szczech (1999) and Szczech and Smolinska (2001) showed a notable decrease in the infection caused by *Phytophthora nicotianae* and *Fusarium lycopersici* in tomato cultivated on soil that had been modified with VC. According to Arancon *et al*. (2005), *Pseudococcus* spp., *Myzus persicae* and *Peiris brassicae* infections are much less common in tomato, pepper and cabbage plants cultivated in VC modified media. Infestations of *Acalymma vittatum*, *Manduca quinquemaculata* and *Diabotricaun decempunctata* in cucumber and tomato plants planted in pig dung VC were reduced, according to Yardim *et al*. (2006).

4. Conclusion

Without proper treatment, industrial pollutants and solid organic wastes may pollute the soil and other wildlife, posing serious health risks. According to the findings of numerous authors, vermitechnology is a useful method for reducing the toxic effects of industrial wastes and solid organic wastes. In vermitechnology, earthworms and microorganisms work together to reduce organic waste and produce the final vermicompost, which has the best physicochemical and biological properties since it has been stabilised and finely split. The majority of research also showed that the vermicompost's end product may function as an appropriate medium for plant growth since it has a higher concentration of soil enzymes and growth hormones. Earthworm activity results in the production of vermicompost, which is high in macro- and micronutrients, growth hormones, vitamins, and enzymes like amylases, proteases, cellulose lyases, lipases and chitinases as well as immobilised microflora. Vermicompost is the best organic supplement for improvement of plant growth and development and output in general. Without harmful consequences towards the environment, it can enhance the crop production rate and shield them from harmful pests. Vermicompost application accelerated growth, enhanced plant nutrition and enhanced fruit and seed quality.

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A comprehensive review on the phytochemistry and pharmacodynamics of *Alstonia scholaris* (L.) R. Br.

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ABSTRACT

Alstonia scholaris (L.) R. Br., a tropical plant belonging to Apocynaceae family had been a fruitful traditional medicine in ayurveda and was used in several ailments. *Alstonia scholaris* is rich in phytochemicals like alkaloids, terpenoids, flavonoids, iridoids and essential oils. These phytochemicals are isolated from roots, stem, bark, fruits, flowers and leaves of *Alstonia scholaris.* The pharmacological aspect of *Alstonia scholaris* shows its greater potential in treatment of bacterial and viral infection, inflammatory diseases, ulcers, rheumatism, diarrhoea, hyperuricemia, diabetes, malaria, neural and cardiovascular diseases. Apart from these, *Alstonia scholaris* have been found to show antioxidant, immunomodulatory, antidepressant, analgesic and anticancer properties including cytotoxicity against various cancers in vitro and in vivo conditions. However, even after showing such pharmacological relevance, the clinical and pre-clinical studies of *Alstonia scholaris* is negligible. This review provides insights to the existing knowledge of *Alstonia scholaris* and its requisite in further research, so that it can be used as a therapeutic drug in near future.

1. Introduction

The role of medicinal plants in traditional medicine and also as raw material in pharmaceutics has been unimaginable. Considering its vast extension, the use of therapeutic plants has been increasing all over the world. The World Health Organizations report states that at least 70% of Indians are regularly using therapeutic plants as traditional medicines for treating several diseases (WHO, 2019). The therapeutic plants have been fundamental to the Indian ethnomedinicity- Ayurveda, Sidha, Unani and Tibetan medicine, as well as other folk medicines. One such medicinal plant, *Alstonia scholaris* (L.) R. Br. *(A. scholaris)* has been known for its conventional uses and excellent pharmacological relevance. *A. scholaris* is an tropical evergreen tree belonging to the family of Dogbanes (Apocynaceae) having white coloured perfumed flowers (El-Fiki *et al.*, 2019). Commonly known the Devil's plant or Blackboard tree, *A. scholaris* was initially named as *Echites scholaris*. Later in 1811, Robert Brown renamed the genus name to *Alstonia* to honour Prof. Charles Alston (Oktavia *et al.*, 2020). The species name scholaris was kept due to its importance in scholastics as it was used to make blackboards in school (Pandey *et al.*, 2020). The other names of *A. scholaris* are Saptaparna, Chatian, Milk wood, Phalagaruda and also known in various names as listed in Table 1 (Oktavia *et al.*, 2020).

2. Morphology and geographical distribution

A. scholaris is an epiphyte with a maximum height of 60 m having greyish brown rough bark, white milky latex, and rooting branches. The tree is rounded in shape with 4-8 whorled dense leaves. The leaves are dark green, thick, obovate to oblanceolate and narrow at the base, while the flowers are greenish white coloured, compact, umbel shaped, numerous and well scented (Figure 1). The flowering of *A. scholaris* in India is during December to March and fruiting is during May to July (Majid and Faraj, 2023). *A. scholaris* is found throughout in India, Sri-Lanka through mainland

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South-East Asia including Nepal, Thailand, Vietnam, Papua New Guinea, Southern China, Indonesia, Malaysia throughout Myanmar to northern Australia and Ocenia Islands (Mahar *et al.*, 2022). It grows in evergreen as well as deciduous forests and even in plains. It can be cultivated in all climatic conditions of India, from tropical to sub-tropical regions with healthy growth at an annual rainfall of 100 to 150 cm, as it favours humid atmosphere (Tripathy *et al.*, 2019). *A. scholaris* flourishes well in red and black alluvial soil while it slugs down in rainy season due to wet soil condition (Bhandary, 2020).

3. Ethnomedical uses

The therapeutic application *A. sholaris* has been well mentioned by the name Saptaparna in the Ayurvedic text "Bhavaprakasha" which was earlier use to cure asthma, bleeding leprosy, phantom tumour, ulcers and gastric issues (Oktavia *et al.*, 2020). It also played vital role in the treatment of rheumatism, wounds, malaria, diabetes, cholera and dysentery. Moreover, *A. sholaris* was used an antipyretic and anthelmintic agent, along with major roles in stomach ache, hepatic diseases, skin rashes, swelling and urinary tract infection (Chhajed *et al.*, 2023). In the Yunnan province of China, *A. scholaris* was known as "Dai" which is used for the treatment of respiratory tract infection (Zhao *et al.*, 2021).

4. Phytochemistry

A. scholaris has been the interest of researchers due to its multi-therapeutic applications as it has rich phytochemical constituents. All the parts of the *A. scholaris* (roots, bark, leaves, stem, fruit and flower) have been chemically investigated by several researchers and found to be identified with flavonoids, alkaloids, iridoids, tannins and steroids (Ali *et al.*, 2021). Some other chemical constituents which were reported in *A. scholaris* includes coumarins, terpenoids, phlobatanins, saponins, simple phenolics, primary metabolites and secondary metabolites (Ali *et al.*, 2020). Till now more than 400 phytochemical constituents have been isolated and characterized from *A. scholaris* (Baliga, 2012). Majority of the constituents include alkaloids that are isolated from all parts of *A. scholaris* like roots, stem, bark, leaves flower and even fruits (Zhao *et al.*, 2021) (Table 2). However, out of all the constituents of *A. scholaris*, few have been found to be very important in terms of therapeutic properties (Figure 2).

5. Pharmacodynamics

5.1. Antimicrobial activity

Macabeo and others reported that the methanol extract of *A. scholaris's* leaves, stem and root bark had an antimycobacterial effect. Using the Microplate Alamar Blue Assay (MABA) at a concentration of 50 g/mL, in-vitro antituberculosis activity inhibits about 89% of *Mycobacterium tuberculosis* H37Rv (Tripathi *et al.*, 2019). This study was conducted to confirm the role of butanol bark extracts in *Mycobacterium tuberculosis* (Zhao *et al.*, 2021). The Luciferase reporter phage (LRP) and an in-vitro assay based upon inactivation of viability by a modus operandi similar to the neutralization assay were used to report the inhibition of *Mycobacterium tuberculosis*. Following six days of incubation, when the butanolic extract was given, the in-vitro bioassay results showed comprehensive susceptibility to the rapid expansion species of *Mycobacterium* as in contrast to control (Goel *et al.*, 2021). Another study reported that bark and leaf extract of *A. scholaris* shows ant-microbial activity against various pathogens of human (Bagheri *et al.*, 2020). Alstoscholarisine K, an indole isolated from gall-modulated leaves of *A. scholaris* has potent anti-microbial activity (Yu, 2021). Leaf extracts of *A. scholaris* also exhibits potent activity towards multi-resistant fungal and bacterial strains (Altaf *et al.*, 2019).

5.2. Antioxidant activity

A lot of investigations have been done on the phytochemical analysis and antioxidant activities of *A. scholaris*. The study discovered that, in comparison to butanolic and ethyl acetate extracts, aqueous bark extracts had the antioxidant activity at the peak level in DPPH and ABTS assays (Goel *et al.*, 2021). Antioxidant property of *A. scholaris* will help human being to fight against various disease and stay young and healthy for longer period. But this important property of *A. scholaris* is not known very well till now. So, this property of *A. scholaris* needs more investigation to help human beings with a better option with no side effects.

5.3. Anti-inflammatory, Analgesic, anti-ulcerogenic and anti-rheumatoid activity

It has been reported the antinociceptive and anti-inflammatory characteristics in *A. scholaris* by various scholars. According to the study, ethanolic extract significantly affects hot plate techniques and lessens the inflammatory response in inflammation induced by carrageenan (Sultana *et al.*, 2020). There was a noticeable antiulcerogenic effect of the ethanolic extracts. The leaves of *A. scholaris* are said to possess antioxidant and antiarthritic qualities by researchers. The results of this research indicate that ethanolic extracts have potent antiarthritic qualities, which may be related to their antioxidant, analgesic, anti-inflammatory and immunosuppressive characteristics (Khyade *et al.*, 2014).

Another study has demonstrated the analgesic, anti-inflammatory and antiulcerogenic qualities of *A. scholaris* fractions. The investigation concluded that although the DCM fraction lacked ulcerogenic properties, it did possess peripheral anti-inflammatory and analgesic properties. The effects of the ethyl acetate fractions were negligible (Banik and Das, 2023). In a study, the anti-inflammatory and analgesic properties of *A. scholaris* has been shown. The authors concluded that the three main alkaloids found in *A. scholaris* leaves—picrinine (Figure 2), vallesamine and scholaricine might have some kind of analgesic and anti-inflammatory effect that acts peripherally (Zhan *et al.*, 2023). In this investigation, the conventional mechanism behind the anti-complementary action of stem bark extracts was demonstrated in in-vitro condition. Treatment for rheumatoid arthritis may be benefited from the anticomplementary action of *A. scholaris* (Kanase and Mane, 2018).

5.4. Anti-viral activity

Researches have shown that *A. scholaris* exhibits potent activity against various viruses. According to a study, the total alkaloid (TA) of *A. scholaris* markedly decreased the production of cytokines and chemokines at the levels of mRNA and protein and significantly blocks replication of virus in A549 cells and U937-derived macrophages (Zhou *et al.*, 2020). Moreover, in A549 cells, TA inhibited the activation of signal transduction triggered by type I interferons (IFN) and pattern recognition receptor (PRR). Crucially, in a deadly PR8 mouse model, TA also improved lung histopathology, decreased viral titer, inhibited the production of proinflammatory cytokines and innate immune cell infiltration and raised the survival rate (Zhou *et al.*, 2020). Moreover, it was found that *A. scholaris* exhibits potent preventive activity against infection of SARS-CoV-2 in Hamster Model of Syrian (Rizvi *et al.*, 2023). More studied needed in this field for better therapeutic option.

5.5. Anti-diarrheal activity

A. Scholaris has been shown to have anti-diarrheal properties by several researchers. According to a study, the methanolic crude extract of *A. scholaris* exhibits spasmolytic and anti-diarrheal properties via blocking the calcium channel (Shah *et al.*, 2010). The results of this research indicate that the anti-diarrheal and spasmolytic effects of *A. scholaris* crude extract may be because of the existence of a compound resembling CCB (Oktavia *et al.*, 2020). The anti-diarrheal characteristics of *A. scholaris* is not well known by now, further investigations are required to see its therapeutic effect in diarrhoea.

5.6. Anti-hyperuricemia

Some studies have revealed that *A. scholaris* also exhibits potent anti-hyperuricemia activity. Interestingly it lowers the levels of serum uric acid in models of mice at the concentration of 100 mg/kg and 200 mg/kg (Hu, 2023). Additionally, it shows better activity in HK-2 cell model enhanced by monosodium urate by enhancing the excretion of uric acid at the dose of 5μM (Hu *et al.*, 2022). Scaffolds triterpenoids extracted from leaves of *A. scholaris* shows anti-hyperuricemic properties in both in-vitro and in-vivo condition (Hu, 2021). There is less research data about the anti-hyperuricemia activity of *A. scholaris* till now, so more research is needed in this area.

5.7. Anti-nociceptive activity

It has been reported that some plants have the potent activity against either nootropic mode or stress but *A. scholaris* is one of these plants having such both activities at a time. Methanol bark extract of this plant exhibits both these properties whereas the leaves ethanol extract possesses anti-anxiety activity without having any sedative or stimulant effects (Khyade *et al.*, 2014). A clinical study taking about 30 patients was also conducted to check the effects of *A. scholaris* on hypertension and in result it was found that it potently reduces the both the systolic and diastolic blood pressure and symptoms of psychological disorders (Khyade *et al.*, 2014). Now-a-days human beings are suffering from diseases related to CNSs and there are limited therapeutic options with higher side effects are available for it. Henceforth, in depth invention of anti-nociceptive property of *A. scholaris* will help human being significantly with less/no side effects.

5.8 Anti-malarial activity

Like other plants *A. scholaris* also has the potent killing activity against malarial parasite *Plasmodium*. Although this study is limited but some are reported. Mostly the bark and leaf extract show potent activity against *P. falciparum*. More specifically the methanol extract of bark shows promising anti-plasmodial activity in comparison to others (Singh *et al.*, 2023). Moreover, not enough data has been reported about the anti-malarial activity of *A. scholaris*. Hence more researches are needed in this field to get an ideal anti-malaria agent using different extracts of *A. scholaris*.

5.9. Antidepressant

A study on antidepression provided an account of *A. scholaris*'s impact on stress and cognitive function in mice. After applying the methanolic bark extracts, they found that

all the stress-induced markers—cortisol, glucose, protein, triglycerides, and cholesterol are normalized (Sarkar *et al.*, 2021). As in current scenario antidepressant therapeutics seeks more attention so in depth study about the antidepressant property of *A. scholaris*, which will help a lot to us.

5.10. Anti-diabetic

The potential for hypoglycemia of *A. scholaris* triterpenes was investigated and documented in a study. The authors found lupeol and betulin (Figure 2) to have hypoglycemic action. Another study reported *A. scholaris* Linn. bark's hypoglycemic activity and antihyperlipidemic effects in diabetic rats produced by streptozotocin (Chhajed *et al.*, 2023). Because of its antidiabetic and antihyperlipidemic activity, the research revealed that the bark of *A. scholaris* has potential effects on lipid profile and may be useful in treating diabetes and related cardiovascular problems (Kanase and Mane, 2018). The antihyperlipidemic and antidiabetic properties of *A. scholaris* leaves were demonstrated by researchers. The study found that in diabetic rats produced by streptozotocin, the ethanolic extract of *A. scholaris* exhibited antihyperlipidemic and antioxidant potential in addition to its antidiabetic effect. Dita was the source of α-glucosidase blockers, as described by the study. It has been reported that an aqueous methanol extract from dried Devil tree leaves exhibits α-glucosidase inhibitory action (Oktavia *et al.*, 2020). In detailed study will give a new therapeutic agent using different extracts of *A. scholaris* in the field of diabetics.

5.11. Anti-bacterial activity

Rapid centrifugal chromatography was used in the preliminary isolation of the bioactivator Logenetin from *A. scholaris*, as described by a study. The authors discussed the separation of logenetin and how it combats both gram-positive and gram-negative bacteria (Qin *et al.*, 2015). Wang *et al.* claim that *A. scholaris* and *Leea tetramera* possess antibacterial qualities. They concluded that *A. scholaris* and *Leea tetramera*'s root bark sections were useless against the fungi they looked at (Wang *et al.*, 2016). However no sufficient data is available till now related to anti-bacterial activity of *A. scholaris*, so in detail study in this area will give new insight into this field.

5.12. Immunomodulatory activity

The medicinal plants show their medicinal property by acting on the immune system of host. Like others *A. scholaris* also exhibits significant immunomodulatory role by acting differentially on the immune system of host. Research has been shown that the combination of alkaloid and triterpenes of *A. scholaris* enhances immunomodulatory action in C57BL/6 mice (Al-Rikabi, 2020). Its bark extract also has an immunomodulatory effect. At lower dose the aqueous extract promotes the cellular immunity while at higher dose it apprehends hypersensitivity reactions (Dangi *et al.*, 2018). In depth research regarding this subject will be benefited to mankind in future to boost immunity.

5.13. Anti-cancer and cytotoxic activity

Cancer is the first line disease in world today. Instead of having many treatment options its cure rate is still in worst condition. This is for therapy resistance and higher side effects. So, to overcome this situation now researchers are emphasizing on plant-based therapies as they have various pharmacological activities including anti-cancer activity and less/no side effects. Like other plants *A. scholaris* also exhibits potent cytotoxicity and anti-cancer activity against various cancers in both in-vitro and in-vivo conditions. It has also the ability of chemosensitization. It has been reported that triterpenoids and sterols isolated from leaf of the *A. scholaris* shows potent anti-proliferative activity against NSCLC (Wang, 2017). Additionally, nor-monoterpenoid indole alkaloids from fruit of *A. scholaris* potently kills the stem cells of glioblastoma (Wang, 2018). Alstoniasidines A (1) and B (2) isolated from *A. scholaris* shows cytotoxicity against stem cells of glioma by promoting caspase-3 mediated extrinsic pathway through enhancing the levels of expression of tumor necrosis factor/TNF-α, interleukin 1/IL-1, and the cleaved caspase-3 and also apprehends the self-renewal property of stem cells of glioma (Wei, 2018). It has also been reported that *A. scholaris* competently regulates the stomach cancer of mice promoted by benzopyrene (Chhajed, 2023). Interestingly hydroalcoholic stem bark extract of *A. scholaris* has the capacity of chemo-modulation in combination with berberine hydrochloride in mice having Ehrlich ascites carcinoma in a concentration dependent manner (Khyade, 2014). In A549 NSCLC cells alkaloids and triterpenoids from *A. scholaris* promotes apoptosis via lowering the levels of expression of pro-casp8 and Bcl-2 and up-regulating the expression of cleaved caspase 8 which leads to cell death (Feng *et al.*, 2013). According to a research, *A. scholaris* exhibits anti-mutagenic and anti-carcinogenic effects on peripheral human lymphocyte culture and albino mice bone marrow cells towards genotoxicity enhanced by methyl methane sulfonate (Ahmad *et al.*, 2016). However, there is no recent advances in the study of anti-cancer property of *A. scholaris.* Numerous studies are required in this area to give a novel and beneficial therapeutic regimen in the field of cancer with no side effects and higher cure rate.

6. Toxicity of *A. scholaris*

There are not much of studies which have reported the toxic potential of *A. scholaris*. However, in a study, the authors checked the acute and sub-acute toxicity of the bark extracts of *A. scholaris* by feeding to the mice. They reported the highest acute toxicity in summer season, while least was reported to be in winter season. Moreover, the sub-acute toxicity was checked at a dose of 120 mg/kg and 240 m/kg. The higher dose was found to be more toxic due to higher concentration of echetamine from *A. scholaris* (Baliga, 2012).

7. Conclusion

A. scholaris is a well-known plant that is used to cure a variety of illnesses in traditional and folk medicine. The plant *A. scholaris* has a wide range of pharmacological activities, and many of its isolated compounds have not been studied for their pharmacological activity. For this reason, it appears important to substantiate the use of this plant for therapeutic purposes by conducting scientific validation of the pharmacological properties of its constituents. The precise mechanisms underlying different pharmacological characteristics remain unclear. For this reason, *A. scholaris* merits considerably more clinical study and research before it can be considered a medication of interest.

Table 1

Common names of *Alstonia scholaris* used in different languages of India

Language	Synonyms of *Alstonia scholaris*
English	Devil's tree, Black board tree, White cheese wood, Chalkwood tree, Milky pine, Milk wood, Pine, Dita bark, Birrba
Hindi	Saittan ka jhad, Chatian, Shaitan ped, Chitvan
Sanskrit	Saptaparna, Phalagaruda, Grahanashana, Madagandha, Grahashi, Kshalrya, Payasya, Jivani, Vishalalvaka, Ayugmapama, Vishamachhda
Oriya	Silgandha, Chhanchania, Chhatiana
Bengali	Chattin
Kannada	Hale, Doddapala
Marathi	Salvin, Santhni
Tamil	Pala, Wedrase, Elilaipillai, Mukumpalei
Telugu	Edakulapada
Gujrati	Saptaparni
Malayalam	Daivapala
Sindhi	Rukattana

Table 2

Phytochemical constituents of *A. scholaris*

Phytochemical Class	Compound	Part of *A. scholaris*	Reference
Alkaloids	5-Methoxyaspidophylline	Leaves	(Rudani *et al.*, 2020)
	5a-Methoxystrictamine	Leaves	(Khyade *et al.*, 2014)
	5-Methoxystictamine	Leaves	(Khyade *et al.*, 2014)
	5-epi-Nareline ethyl ether	Leaves	(Chaudhary, 2022)
	6,7-seco-Angustilobine B	Leaves	(Macabeo *et al.*, 2005)
	17-O-Acetylechitamine	Barks	(Zhao *et al.*, 2023)
	18-Hydroxy-19,20-dehydro-7, 21-seco-uleine	Leaves	(Oktavia *et al.*, 2020)
	19-E-Vallesamine	Fruits	(Khyade *et al.*, 2014)

19-S-Scholaricine	Fruits	
19-E-Picrinine	Fruits	
19-E-Akuammidine	Fruits	
19-Epischolaricine	Leaves	(Zhao *et al.*, 2021)
19,20-(E)-Valllesamine	Leaves	(Khyade *et al.*, 2014)
19,20-Dihydrocondylocarpine	Leaves	(Alvi and Muzaffar, 1986)
19,20-E-Alstoscholarine	Leaves	(Oktavia *et al.*, 2020)
20(S)-Tubotaiwine	Leaves	(Jeet *et al.*, 2020)
Akuammicine	Roots	(Mahar *et al.*, 2022)
Akuammicine-N_b-methiodide	Roots	(Reddy, 2016)
Akuammicine-N_b-oxide	Roots	(Haridas *et al.*, 2016)
Akuammicine N-oxide	Barks	
Akuammiginone	Barks	(Salim *et al.*, 2004)
Alstonine	Leaves	(Bainsal *et al.*, 2021)
Alschomine	Leaves	(Qin *et al.*, 2023)
Akuammidine	Leaves	(Mahar *et al.*, 2022)
Angustilobine B N^4-oxide	Leaves	(Krishnan *et al.*, 2019)
Angustilobine B	Leaves	
Angustilobine B acid	Leaves	
Echitaminic acid	Barks	(Rudani *et al.*, 2020)
Echitamine	Barks	
Echitamidine N-oxide	Barks	
Echitamidine	Leaves	
Lagunamine	Leaves	(Lee and Sperry, 2022)
Losbanine	Leaves	(Majid and Faraj, 2023)
Manilamine	Leaves	(Elshaier *et al.*, 2022)
N^4-Methyl angustilobine B	Leaves	(Macabeo *et al.*, 2005)
N^1-Methoxymethyl picrinine	Leaves	
N^b-Methylscholaricine	Leaves	
Nareline	Leaves, Fruits	(Chaudhary, 2022)
Nareline methyl ether	Leaves	(Shrivastava *et al.*, 2016)
Picralinal	Leaves	(Qin *et al.*, 2023)
Picraline	Leaves	(Paul *et al.*, 2021)
Picrinine	Leaves, Flowers, Fruits	(Li *et al.*, 2019)
Quinoline	Leaves	(Yang *et al.*, 2015)
Rhazimanine		(Rudani *et al.*, 2020)
Scholarisine A	Leaves	(Zhan *et al.*, 2020)
Scholaricine	Leaves	
Strictamine	Flower, Fruits	(Hamdiani *et al.*, 2018)
Tubotaiwine oxide	Leaves	(Zhang *et al.*, 2023)
Vallesamine N^b-oxide	Leaves	(Mohammed *et al.*, 2021)

Terpenoids and Sterols	3,28-β-Diacetoxy-5-olea-triterpene		Flower (Dey, 2011)
	β-Sitosterol	Leaves	(Ghansenyuy *et al.*, 2023)
	β-amyrin	Flower	(Akbar *et al.*, 2020)
	n-Tetracosane	Leaves	(Singh *et al.*, 2020)
	α-Amyrin acetate	Barks, Flowers, Fruits	(Ali *et al.*, 2022)
	Alstonic acids A and B	Leaves	(Akbar, 2020)
	Betulin	Leaves, Flowers	(Ali *et al.*, 2021)
	Betulinic acid	Leaves, Flowers	(Akbar *et al.*, 2020)
	Lupeol acetate	Barks	(Ali *et al.*, 2021)
	Oleanolic acid	Leaves	(Wang *et al.*, 2017)
	Sweroside	Leaves	(Zengin *et al.*, 2023)
	Ursolic acid	Leaves, Flowers	(Wang *et al.*, 2017)
Flavonoids	Quercetin	Leaves	(Banik *et al.*, 2023)
	Quercetin-3-O-β-D-galactopyranoside	Leaves	(Banik *et al.*, 2023)
	(+)-lyoniresinol 3α-O-β-D-glucopyranoside	Leaves	(Afreen *et al.*, 2021)
	Kaempferol	Leaves	(Singh *et al.*, 2017)
	Isorhamnetin	Barks	(Kawiwong *et al.*, 2020)
	Apioglucosides	Stems	(Chanda and Ramachandra, 2019)
	Isorhamnetin-3-O-β-D-galactopyranoside	Leaves	(Bainsal *et al.*, 2021)
	Kaempferol-3-O-β-D-galactopyranoside	Leaves	(Singh *et al.*, 2017)
	Alstonoside 1	Stems	(Nanditha *et al.*, 2020)
Iridoids	Scholareins A-D	Barks	(Indradi *et al.*, 2023)
	Isoboonein	Barks	(Khyade *et al.*, 2014)
	Alyxialactone	Barks	(Khyade *et al.*, 2014)
	Loganin	Barks	(Feng *et al.*, 2008)
	Loganetin	Stems	
Essential oils	α-Terpineol	Flowers	(Singh *et al.*, 2020)
	Terpinen-4-ol	Flowers	
	Linalool	Flowers	
	2-Phenylethyl acetate	Flowers	
	Furanoid	Flowers	
	Pyranoid	Flowers	

Alstonia scholaris plant

Leaves of *Alstonia scholaris*

Bark of *Alstonia scholaris*

Flower of *Alstonia scholaris*

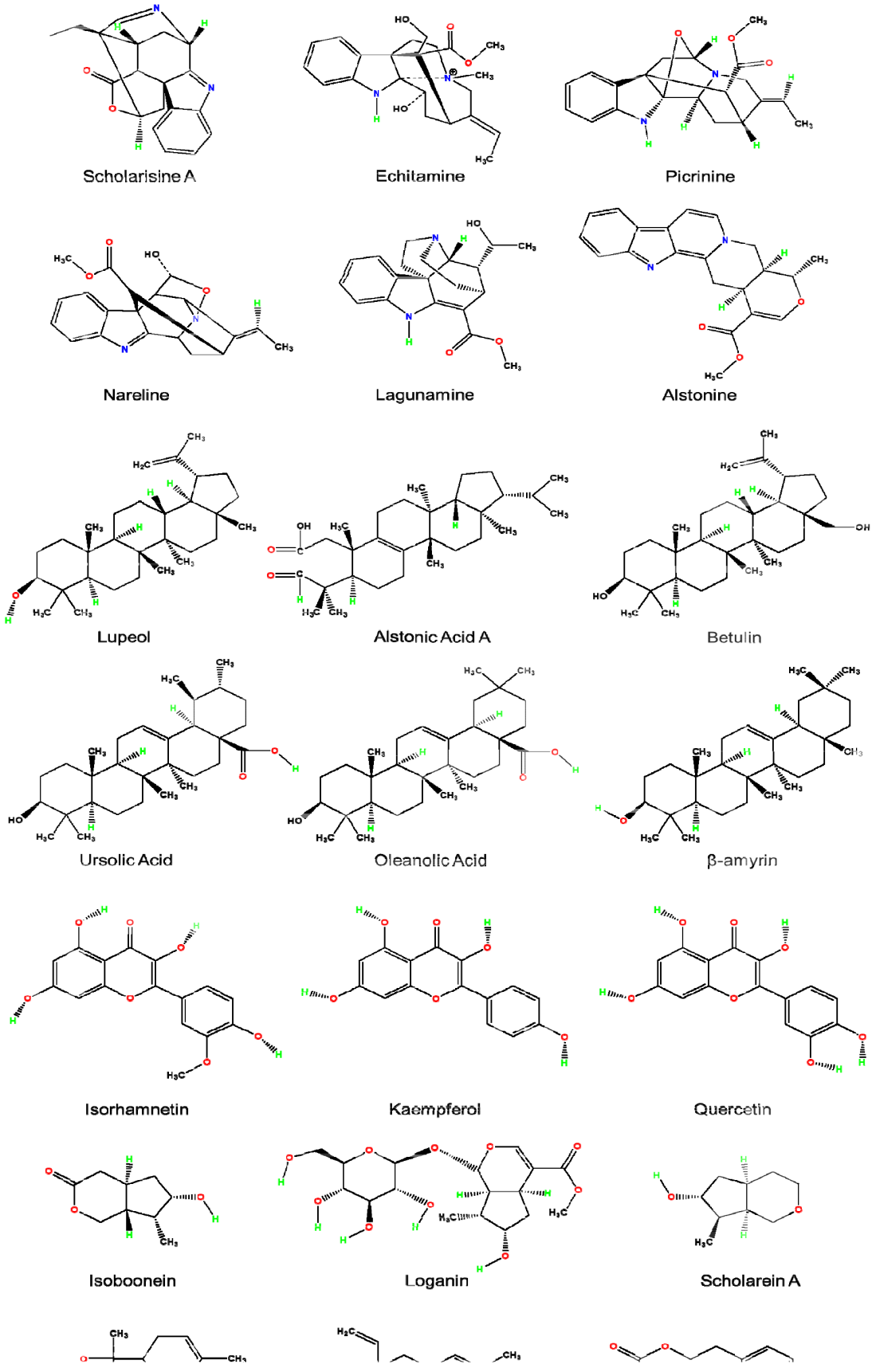

Figure 2: Structure of some phytochemicals isolated from *Alstonia scholaris*

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Artemisia: Sweet worm wood used for folk remedies

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ABSTRACT

Artemisia genus comprises more than 2290 species that have been recorded. The genus *Artemisia* includes grassy and bushy plants with variable habitats. These small aromatic herbs were distributed almost in all continents except Antarctica. These plants are part of different ecosystems, but mostly of temperate zone. Species of *Artemisia* are distributed widely all over the world. Though, *Artemisia vulgaris* L. is native to Europe and Asia, however it has been located in Africa, North America, Himalayas and Australia. Some *Artemisia* species are become invasive due to easy adaptation to a new habitat and always remaining in RET category. The examples are *Artemisia princeps* Pamp., which is native to Japan, China and Korea but invasive in Belgium and Netherlands and *Artemisia verlotiorum* L. is also invasive in Croatia. The *Artemisia* species is used in traditional medicine and this genus has great ethno-pharmacological value. *Artemisia annua* L. is locally known as "Sweet worm wood" which has reported to be used for treating disease and symptoms like food borne fever, Jaundice, summer worts, tuberculosis, lice, scabies, dysentery and hemorrhoids. *Artemisia nilagirica* (C.B.Clarke) Pamp. locally known as "Indian worm wood" which has been used for treatment of inflammatory diseases, malaria/ hypoglycemia, stress related depression and other microbial pathogenic disorders.

1. Introduction

India is one of the largest mega bio-diverse country in the world. Its civilization is very ancient and, the country as a whole has long been known for its rich resources of medicinal plants. Today, Ayurvedic, Homeo and Unani physicians utilize numerous species of medicinal plants that found their way a long time ago into the "Hindu Materia Medica". According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Use of herbal medicines in Asia represents a long history of human interaction with the environment. Plant used for traditional medicine contain a wide range of secondary metabolites that can be used to treat chronic as well as systemic disease. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance. One of such resources is folk medicine and systematic screening of the plants in the discovery of novel effective metabolites.

Artemisia absinthium L. is widely used to treat gastrointestinal disease, anti-parasitic, anti-hypertensive, anti-pyretic, and anti-inflammatory. *Artemisia afra* Jacq. is mostly used as herbal remedies to treat pain, cough, colds, inflammation, asthma, fever, influenza, diabetes, and malaria. *Artemisia vulgaris* L. "Mugwort or wild worm wood" used to treat gonorrheal sore, cold, headache, rheumatism, steam bath for pleurisy, pains of after birth. In Europe the *Artemisia* species are mainly used as food, spices and beverages. As per the traditional Mongolian and Chinese medicine records *A. ordosica* Krasch has many beneficial effects on the nasal bleeding, headache, sour throat and carbuncle. In Tuscany folk medicine *A. verlotiorum* L. is used for the reduction of hypertension.

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Reports on drug preparation, uses and present scenario

Artemisia *species in food industry;*

Artemisia as a grassy and bushy herb of family Asteraceae is edible with multi-nutrients. This herb is used as spices, beverages and condiments in most Asian countries as well as North America. The following table is the enumeration of *Artemisia* as food, spice and flavoring agent.

Many *Artemisia* species have been used as food. For example, the leaves of *A. vulgaris* L. are used for preparation of two different rice cakes, kusa-mochi andhishi-mocha. Shoot tips, leaves and stem of *A. dracunculus* L.*, A. japonica* Thunb.*, A. vulgaris* L. can be eaten directly as salad or used as any other food supplementation. The seeds of *A. dracunculoides* L. and *A. tridentata* Nutt. used in roasted form; powdered; to be used in raw or along with water. Several *Artemisia* species are used in preparation of different

Table-1

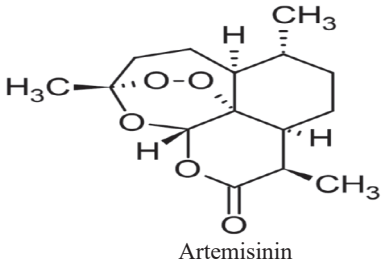
Distribution and uses of *Artemisia* species

Species	Distribution	Edible part	Uses
A.abrotanum L.	South Europe	Young shoots	Flavoring cakes, salads, and herbal tea
A.absinthium L.	Europe, Asia	Whole plant	Flavoring beer, wine, vermouth, liquors
A.afra Jacq.	Africa	Whole plant	Flavoring, preparation of vermouth, as a tea
A.annua L.	South-East Europe to Asia	Leaves	Vermouth, as a vegetable
A.capillaries Thunb.	China, Japan, Korea	Leaves, stem, shoots	Soaked and boiled then eaten as food Supplement's in time of famines
A.indica Willd.	India, Japan and China	Leaves	Young leaves are cooked along with barley
A.japanica Thunb.	Korea, Japan, China	Young leaves	For cooking
A.maritima L.	Eastern-Asia, Europe	Leaves	Flavoring of Beer and liquor
A.nilagirica (C.B.Clarke) Pamp.	India	Stem and Leaves	Herbal tea
A.vulgaris L.	Europe and Asia	Leaves, flowering top	Flavoring in beer

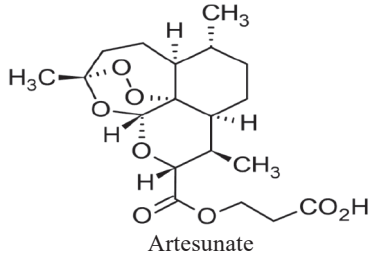
tonic, beverages, beer and vermouth. Thus *A. absinthium* L.*, A. vulgaris* L. and *A. maritima* L. have been used as flavoring ingredient in beer production. A special kind of alcoholic drink, the absinthe and vermouth is prepared from *A. absinthium* L..Vermouth is a low alcoholic drink (Morata *et al.*, 2019). In Switzerland the spirit drink absin the was created by macerating leaves of *A.absinthium* L. with seeds of fennel and alcohol.

Artemisinin and its derivatives:

Potential terpene lactone which was discovered by Tu from *A.annua* L. *as* anti-malarial drug in 2015 brought him Nobel Prize in 2017. This terpene has a short half-lifeand first passage metabolism with sparingly soluble in water and oil (Letchmanan *et al.*, 2018). By reducing the lactone producing a potent hemi-acetal, these are dihydro-artemisinin and artenimol. After alkylation of hemi-acetal arte-ether and arte-mether was generated but artesunate was produced by acylation of the hemi-acetal with succinic acid. *Artemisinin* and its derivative have been widely used for anti-malarial activity and also in treatment of leishmaniasis, trypanosomiasis and schistosomiasis. This terpene was also used for anti-ulcerous, antinociceptive, antifungal, antiviral and antibacterial activities.



Artemisinin



Artesunate

Biological activities

(i) Anti-parasitic activities: Mosquito–vectorial diseases are one of the most uncontrollable deadly diseases, can be partly control by artemisinin combination therapy (ACT) as directed by the world health organization. Derivatives of artemisinin 1, 2, 4-trioxane moiety which is reported to be the main active component in artemisinin (Wang *et al.*, 2019). *A. annua* L. decoction provides sufficient amount of anti-plasmodial activity. Besides the decoction the capsule or tablets that are produced from powdered leaves of *A. annua* L. have excellent antimalarial activity. The extracts from *A. absinthium* L. shows anti-protozoal activity against *Trypanosoma brucci, T. cruzi, Plasmodium falciparum, Trichonema vaginalsis, Leishmania donovani and Entamoeba histolytica*. The ethanolic extract has the best inhibition effect of 96.2% against protozoa. The essential oil which was isolated from *A. absinthium* L. is used against *Leishmania aethiopica and L. donovani*. *A. nilagirica* (C.B. Clarke) Pamp. containsmany essential oils and other chemical compounds which are used to examine the larvicidal activity. It is concluded that artemisinin significantly act against *Aedes albopictus* the tiger mosquito of South-East Asia.

(ii) Antifungal activities: In recent times fungal infection has been reported in immune suppressed patients, suffering from endemic diseases like AIDS and cancer, organ or tissue transplantation and stem cell remedies. In *A.nilagirica* (C.B. Clarke) Pamp. essential oils can be used to control phytopathogenic fungi infecting agricultural crops and commodities. Extract from *A.annua* L. is used against fungi like *Candida malassezia* and pathogenic *Saccharomyces* species. *A. absinthium* has highly effective antifungal remedies. Several reports revealed the effectiveness of *A. annua* L. extracts against a plethora of microorganisms, this includes fungi such as *Aspergillus niger, Candida albicans.*

(iii) Antimicrobial activities: *A. annua* L. exhibits effective antimicrobial activities. The aerial parts and seeds of *Artemisia annua* L. unusually rich with essential oil like 1, 8-cineole, Camphor, Trans -3 (10)-caren-4-ol. which have significant anti-microbial activity. The extracts of *A. absinthium* L. are particularly effective against gram positive bacteria. As the protective phospho-lipid layer (Gram –ve) has greater resistance against antimicrobial agents. In 2011, international studies determined the antimicrobial potential of *A. absinthium* L. It has been marked for a metabolite i.e., caffeoylquinic acid, effective against gram +ve like *S. aureus, E. faecalis, E. coli and C. albicans* (fungi). Two active components of *A. absinthium* L., chlorogenic acid with low antimicrobial activity and the other is 4,5–di-O-caffeoylquinic acid which inhibits pump activity of Gram positives. The essential oil of *A.absinthium* L. has been used against bacteria like *Anthrobacter* spp., *Bacillus mycoides, Micrococcus lylae, Pseudomonas aeruginosa.* The methanol extract of *A.nilagirica* (C.B. Clarke) Pamp. exhibits maximum activity against *Bacillus subtilis*, *Enterobacter aerogenes*, *E.coli, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri* and *Yersinia enterocolitica*.

(iv) Antiulcer activity: In the modern world peptic ulcer is a major concern of civilian society due to junk food and unhealthy easy going life style. *A. nilagirica* (C.B. Clarke) Pamp. is a rich source of essential oils which has potential antiulcer property. Limonene obtained from *A. nilagirica* (C.B. Clarke) Pamp. used as flavoring agent in beverages and food which reduces the toxicity of other additives. The essential oil of *A. nilagirica* (C.B. Clarke) Pamp. also have gastro-protective activity (Rozza *et al.*, 2011). The extracts of *A. nilagirica* (C.B. Clarke) Pamp. used as a protectant against ulcer induced disease and increase the mucus content. Extracts of *A. absinthium* L. obtained by using various polar and non-polar solvents like ethanol, methanol, hexane, chloroform and carbon tetra-chloride were administered on rodents, both and after they had received acetylsalicylic acid. The extract couldn't affect the secretion of gastric juice but decreases the secretion of pepsin, gastric acid and a decrease in digestion rate by reducing the quantity of gastric juice. *A. annua* L. contains three sesquiterpene lactone like artemisinin, dihydro-epideoxyarteannuin-B and deoxyartemisinin which exhibited anti-ulcergenic activities by increasing prostaglandin level in gastric mucosa.

(v) Anti-inflammatory activity: Artemisinin as an anti-inflammatory agent have been investigated on autoimmune disease, septic and allergic hypersensitivity. Effective anti-inflammatory property of artemisinin evidenced mitogen activated protein kinase (MAPK), P13K/AKT signaling cascade, TLR9 and NF- KB activation (Wang *et al.*, 2017). The aqueous extracts of *A. annua* L. have inhibited inflammatory activities. *A. annua* L.has major components like artemisinin, scopoletin, eupatin, casticin, chrysophanol, rosmarinic acid, 3-O-β-D- glucopyranoside of sitosterol that exhibit anti-inflammatory activity. The methanolic extract of *A. absinthium* L. exhibits significant anti-inflammatory effect. Similarly anti-inflammatory activity was also observed with different concentrations of aqueous extract and essential oil of *A. absinthium* L. Cardamonin is a chalcone analog isolated from *A. absinthium* L. has profound effect on two cell lines THP-1 and RAW-264.7 by inducing release of nitrites. Cardamonin was also experimentally analysed for its activation of the pathways of MAP kinase like ERK, JNK, P^{38}MAP kinases and NFKB pathway. *A. absinthium* L. has

reduced inflammation by inducing carrageenan and the venom of *Montivipera xanthina* in rats. *A. annua* L. extracts, 25 and 50 mg/kg has been quite effective for treatment of edema. However, 12.5, 25 and 50mg /kg was effective for carrageenan induced inflammation.

(vi) Anticancer activities: Artemisinin and its derivatives have anticancer properties via inducing tissue invasion of tumor, promoting apoptosis, arresting the cancerous cell cycle and a checkpoint for angiogenesis. *A. annua* L. exhibited anti-cancer activity by inducing reduced mitochondrial membrane potential, imbalancing cell glucose metabolism, reducing VCAM1-1 expression, arresting G1 and G2/M phase of cell cycle and inhibiting MMP-2, MMP-9 and EMT. Artemisinin is stimulatorily effective for cancerous cell lines. Besides Artemisinin other components from *A. annua* L.like flavonoid 6,7,3,4,- tetra methyl ether having toxicity against P-388, A-549 , 4T-29 , MCF- 7 and KB cell line of tumors. The extracts of *A. annua* L. contains Chrysoplenol-D, Artemannuin-B and Castic in which vehemently opposed the cell growth and proliferation in tumor cells. This analysis helps in triggering apoptosis and inhibiting tumor growth (Lang *et al.*, 2019). The dried leaf of *A. annua* L. possesses potential anti-cancer activity. It has also been reported that Artesunate has profound effect on lung cancer like artemisinin, (Raesias and Weather 2019). Two flavonoids, casticin and chrysoplenol-D possess anticancer property for other type of sarcoma, carcinoma or myeloma. The first one Casticin, a poly-methoxy flavone commonly obtained from other herbal drugs. However, the amount of casticin in *A. annua* L. is approximately, 1.07±0.23mg/g while Chryosplenol-D is approximately 0.64±0.14 mg/g (Fu *et al.*, 2020).

A. nilagirica (C.B. Clarke) Pamp. is dramatically active against cancer induced mice. Especially the ethanol and methanolicextract of *A. nilagirica* (C.B. Clarke) Pamp. was active against cancerous albino mice.

(vii) Anti-oxidant activities: The antioxidant activity of *A. absinthium* L.with rich flavonoids and phenolics was estimated by DPPH radical scavenging assay. Methanol (70%), petroleum ether, chloroform, ethyl acetate and n-butanol were used as solvents for extraction. However ethyl-acetate, methanol, n-butanol, chloroform and ether as solvent were more potent for antioxidant activity. Quercetin 12.4 mg/g equivalent and Gallic acid 194.9 mg/g equivalent were obtained from methanolic extract of *A. absinthium* L.. It has shown an educational potential to be conjugated with iron (iii), ions for chelation of iron. The test proved that the flavonoid and phenol found in *A. absinthium* L. has effective antioxidant activity. The extract of *A. absinthium* L. from Spanish crops has a stronger antioxidant effect than the individual flavonoids by DPPH radical test. The individual test for flavonoids like artemisin, casticin and hydroxyl-pelenolide, evidenced that hydroxyl pelenolide has been shown to have a stronger antioxidant effect than others. As a result it was observed and reported that antioxidant activity of *A. absinthium* L. is a synergistical reaction of the phenolic profile present in the plant extract. In the various region of Tunisia, the methanolic extract of *A. absinthium* L. herb was tested by using the DPPH method which showed antioxidant activity. The results indicated that location of habitat from which the plant was harvested related to effectiveness of the antioxidant activity. Plants collected from northern part of the Tunisia have strongest antioxidant potential (IC50=9.38mg/ml) (Msaada *et al.*, 2015). The essential oils of *A. absinthium* has significant antioxidant properties evidenced by DPPH and ABTS 2, 22 -Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) methods. The essential oils of *A. nilagirica* (C.B. Clarke) Pamp. also reported with maximum DPPH radical scavenging property which helps to

Table 2

Anti-cancer activities of *Artemisia annua*

Compounds	Cancers	Subjects	Effects
Polysaccharides	Hepatoma	Tumor xeno graft Mice induced by mouse Hepatoma H22cells	HQG inhibited tumor growth in a dosedependent manner. Increase the cell Apoptosis rate.
A. annua L. methanolic extract	Acute lymphoblastic leukemia	Acute lymphoblastic leukemia cell lines Nalm-6 and Reh	AAME exerted time and dose dependent cytotoxic effect increased them RNA expression Levels of caspase-3 and Bax
Polyphenol	Breast cancer	Human breast cancer cell line MDA-MB-231	PKAL inhibited MDA-MBA-231cells in a dose dependent manner, inhibition of EM
Powdered dried Leaf of *A.annua* L.	Non-small cell lung cancer	NSCLC Cell lines A549, H12990 and PC9	DNA damage G2/M cell cycle arrest.

scavenge radical related to cancerous cell growth (Tripathy *et al.*, 2015).

(viii) Anti-asthmatic activities: The respiratory tract diseases like asthma is mainly caused by common allergens present in the environment (Cazzoletti *et al.*, 2010). Microbial load in the respiratory tract or any other allergen causes asthma in children. Most of the times bronchial asthma was prevalent due to generation of free radicals. However free oxygen radicals penetrate the membrane metabolites like lipid, protein or their glycans. The pathogen of asthma reacts with reactive oxygen species that induce bronchial hyper reactivity, promoting release of histamines from mast cells and mucous secretion from airway epithelial cells. The aqueous extracts of *A. nilagirica* (C.B. Clarke) Pamp. at a concentration of 200 mg/kg shows significant activity, Chloroform extract of *A. annua* L. has been inhibited high K^+ induced contraction on dose dependent manner (IC50=0.316mg/ml), anti-asthmatic activities. *In vitro* investigation on anti-asthmatic activities of *A. annua* L. was reported by using tracheal rings and acute isolated airway smooth muscle cells of mice (Aparna *et al.*, 2009).

(ix) Cytotoxic effect: The methanolic extract of *A. absinthium* L. was effective against breast cancer cell lines, MDA –MB 231 line (non-oestrogen responsive) and MCF–7 breast adeno carcinoma cells oestrogen responsive. The treatment evidenced 50% inhibition of MDA-MB231 cells proliferation at 20g/ml and 50% inhibition of MCF-7 cells at 25g/ml. The above results indicate that *A. absinthium* L. has significant contribution which inhibits development of breast cancer and breast adeno carcinoma. *In vitro* assessment of anti-cancerous activities with essential oils of *A. absinthium* L. was analyzed for six cell lines-A548 (lung adeno carcinoma), NCI-H292 (Non-small cell lung cancer), HCT116 (Colon cancer), MCF-7 (Breast adeno carcinoma), SK-MEL-5 (Melanoma) and HS5 (Bone marrow stromal cell). An observable anti-cancerous activity was noticed against SK-MEL-5 and HCT116 cell lines with least against the MCF-7 lines. The noticeable cytotoxic effect was due to the presence of caryophyllene and germacrene D with higher amount in the composition of essential oil (Martinez *et al.*, 2015).

Application in cosmetology: Along with biological activity, *A. absinthium* L. has been an additive for the preparation of cosmetics used for scalp, face and hair care. According to Cosmetic Ingredient database (Cosing) a European database gathering data on cosmetics ingredients that allows the use of *A. absinthium* L. in five forms. These are skin care products with antibacterial activity and fragrance (Cosing-accessed). The raw materials obtained from plants are used as concentric products such as shampoos, masks, essence, tonics, under eye patches and moisturizing creams with SPF filters. These forms of components used in cosmetics to protect, moisturize and cleanse the skin. These components are produced mainly with extract of the plant or distilled oil. After fermentation of leaves by *Lacto bacillus* species, filtrate was used for the production of different cosmetology components. The products containing absinthium found in foreign companies worldwide, amongst which South Korea, Russia and America cosmetic producers are leaders.

Artemisinin and its derivatives have been used for anti-inflammatory properties and to increase skin immunity. The extract of *A. annua* L. has been used in cosmetics to significantly repair the damage of skin barrier function, reduce the degree of skin redness and improve the condition of sensitive skin.

Application in Therapeutic uses: For treatment of various diseases *Artemisia* species were always used traditionally and research on their pharmacological effects, supports the therapeutic applications. *Artemisia* possesses a lot of properties like anti-malaria, anti-oxidant, cytotoxic, neuro-protective, anti-inflammatory, anthelmintic and antimicrobial. The supplementation of food material to pets with *A. annua* L. preparation helps in treatment of tumors

Table 3

Use of *Artemisia absinthium* in cosmetology

Plant	Function	Plant Part
A.absinthium L. whole plant extract	Skin conditioning	Extract from whole worm wood herb
A.absinthium L. whole plant extract	Perfuming	Extract from blooming herb of worm wood
A.absinthium L. oil	Antimicrobial	Volatile oil obtained from whole plant
A.absinthium L. leaf extract	Skin conditioning	Obtained by fermentation of leaves by bacteria of the genus *Lactobacillus*
A.absinthium L. herb oil	Perfuming	Senti Essential oil obtained from whole worm wood plant

related to veterinary science (Saeed *et al.*, 2020). In traditional Chinese medicine *A. annua* L.was used for curing fever, malaria, inflammation. However, it was also used in the repairment of arthritis of hip and knee joint pain management, heterophyid infection and curing of mosquito vector diseases. *A. dracunculus* L. remarkably used for insulin sensitive glycemic control, secretion of insulin, while *A. princeps* Pamp. used to control mild type- 2 diabetes and *A. absinthium* L. used to control insulin dependent diabetes. *A. absinthium* L. is particularly suppresses the activity of TNF-α and other Leukotriens used for arthritis of knee joints (Basiri *et al.*, 2017). Cardamonin is one type of component that is present in plants responsible for anti-inflammatory activity. *A. vulgaris L.* is used to treat itching in icteric and dialyzed patients with antihistamine and anti-allergenic effects and also used as lotion to partially cure hypertrophic scars generated by burning. Sometimes *Artemisia annua* L. and *A. vulgaris L.* produced the highestlevel of allergens in their pollen which is a cause of allergic rhinitis. The extract of *A. ordosica* Krasch. used to control the allergic inflammatory response in rhinitis while *A. abrotanum* L. contains essential oil and flavonoids used as nasal spray preparation. *A. afra* Jacq. and *A. annua* L. contain artemisinin derivatives, artesunate and amodiaquine respectively used to treat malaria by infusion process. *A. annua* L. extract was used to repair sensitive skin, inhibit inflammation, repair damaged skin, and also reduces redness and other sensitive skin allergies. Beside this, *A. annua* L.fresh leaves are used as salads in some Asian and United states and its ground leaf extract used as direct supplements (Askarya *et al.*, 2020).

Adverse effects of *Artemisia* species: Pollinosis is a serious allergic disorder and most frequent in many parts of the world due to pollen variations in *Artemisia.* In recent research the observation of pollen allergen by nasal passage occurs not only by pollen but also by leaves and stems. *Artemisia* pollen can accelerate allergic rhinitis, along with asthma, or both (Gao *et al.*, 2019). *A. vulgaris* L. pollen contains allergic substances with IgE reactivity, which induce hypersensitive type I allergic reaction like anaphylactic shock. In Europe it was detected that the pollen was collected from *A. vulgaris* L. contains highest levels of endotoxin (Oeteros *et al.*, 2019). The spread of pollen of these species *A. campestris* L., *A. annua* L. and *A. verlotiorum* L. could affect human health, mostly the allergenic pollen dehisces and spread in autumn. The pollen when comes in contact with the skin reacts to generate hypersensitivity. About 43 % patient with allergic rhinitis and asthma attack has positive reactions to Mugwort on skin prick testing. In the year 1910-1920 many countries have prohibited consumption of absinthe because their consumption associated with reactive symptoms like absinthism, blindness, hallucination, convulsions and mental retardation. European parliament and council constituted a regulation for use of *A. absinthium* L.in foods and alcoholic beverages (European parliament and council 12th Oct 2020). While European Food Safety Authority (EFSA) regulates that preparation of absinthe should not exceed the addition of thujone amount 10 mg/kg (Community Herbal Monograph). Some *Artemisia* species such as A. *annua* L., *A. vulgaris* L*., A. herba- alba* L*., A. arborescens* L. *and A. douglasiana* Bess. used for regulation of fertility but not at the time of pregnancy (De Boer *et al.,* 2014). The hydroalcoholic extract of *A. kopetdaghensis* Krasch. was used to treat pregnant rat from 2nd to 8th day of pregnancy. Abortion occurred due to high content of camphor which crosses the placenta.

Hotpoint research of *Artemisia* species used for treatment of COVID -19 infections: WHO (World Health Organization) declared a global health emergency for COVID-19 that belongs to the SARS family to threaten life on earth. The first case of COVID-19 infection was reported on January 27, 2020 in Kerala, India. It causes multisystem disease leading to death. To treat infection, WHO had proposed *Artemisia annua* L.which is a traditional Chinese patent herb having 13 therapeutic names (Lis *et al.* 2005). The extraction of *A.annua* L.inhibited cytopathy of SARS-CoV and showed activity against SARS CoV -2.

The review presents an overview of *Artemisia* species that are traditionally used as medicine, food, herb, tea, beverage, spices, and condiments. The plant is mainly used in flavoring food, tea, salads, while leaves and aerial parts are also used as edible materials. Some studies published the adverse effect of *Artemisia*, that cause pollen allergy, dermatitis and allergic rhinitis. Mostly sesquiterpene lactones artemisinin and its derivatives showed adverse effects. Well known drink absinthe causes adverse side effects and contains a high amount of thujone. *Artemisia afra* Jacq. causes reduction in fertility. The plant *Artemisia* has paramount pharmacological importance. It has been used to cure malarial, fungal, bacterial infections as well as in controlling allergic activities. WHO declared artemisinin and its derivative from *A. annua* L. to be used for treatment of the pandemic COVID- 19 after purification of the herb.

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Integrated approach for selective utilization of phytobiomass: efficient phytoremediation

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ABSTRACT

Natural resources, especially soil and water bodies which are near to mining area or industrial area are most polluted with toxic heavy metals since industrial revolution. The persistent heavy metals contaminations becomea threat to 'Man and Biosphere'. Implementing sustainable practices such as phytoremediation contributes to mitigating the ongoing threat of heavy metals by reclaiming these contaminants from polluted soil. Recently, naturally occurring hyperaccumulator, tolerant species and transgenic plants are used for heavy-metal extraction. Extensive research is focusing on phytoremediation using plants like *Pteris vittata*, *Ricinus communis*, *Jatropha curcas*, and *Cannabis sativa,* Brassicaceae, Asteraceae, to extract heavy metals from the soil. Choosing an integrated system is crucial, where plant species act as hyperaccumulators and their biomass is utilized for purposes like biofumigation, biofortification and bioenergy production. Despite of certain limitations, the phytoremediation is one of the most efficient and cost-effective technology for reclamation of heavymetal from soil. Thus, the review mainly focuses on some known hyperaccumulator selectively utilized in field of biocides, biogas and nutrient enrichment of crops and biochar production for efficient phytoremediation.

1. Introduction

In recent years, it has been a great concern on ecological and global public health which is associated with environmental contamination by heavy metals due to rapid increase in industrialization, agricultural practices and the mining activities. (Sandeep *et al.*, 2019; Wang *et al.*, 2017). Heavymetals are the metallic element characterised by high densities to water (atomic mass greater than 20) and due to their persistence nature, they are considered as global pollutants. There are two types of metals either essential for plant or non-essential. Elements including cobalt (Co), copper (Cu), iron (Fe), nickel (Ni), manganese (Mn) and zinc (Zn) are basic heavy metals that are considered as essential micronutrients but can become toxic when present in excessive amount. On contrary, cadmium (Cd), mercury (Hg) and lead (Pb), chromium (Cr) are non-essential heavy metal that have adverse effect on living organisms even at low concentration and have no role in plant metabolism (Sandeep *et al.*, 2019). Various sources, such as industrial waste, surface runoff, human activities, mining, fossil fuel combustion, automobile exhaust, industrial processes and the cultivation of vegetables in contaminated areas, collectively contribute to the deposition of heavy metals in the biosphere.

The metal pollution has very much impact on biological system as it does not undergo the process of biodegradation. Accumulation of these metals in food chain result in biomagnification and it adversely affects the ecosystem. Heavy metal not only exert a significant impact on fauna and flora but also have consequences on the soil nutrient profile, (Sandeep *et al.*, 2019). Further more, it also inhibits photosynthesis, enzymatic activity and cellular integrity, indirectly reduced the growth and development of plant. People are encountering heavy metal by inhalation,

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drinking water, eating fish, sea food and using cosmetics or any other manmade things in day-to-day life. Although some metals essentially required for various metabolic activity in body. However higher exposure to these heavy metal leads to various negative health impacts. Metals like lead is highly toxic for human especially to children, arsenic associated with respiratory disorder, lung infection and cardio vascular disorder. Chromium is the potent carcinogen and nickel is very dangerous as it causes lungs cancer, allergies, various kidney, and heart related disease. Exposure to cadmium also causes various diseases in human beings (Loh *et al.*, 2016; Coetzee *et al.*, 2020; Genchi *et al.*, 2020). However, in biosphere the amount heavymetal deposition increasesrapidly.

Though characterization of Physio-chemical parameters like precipitation, reverse osmosis, heat treatment, ion exchange, soil washing, solidification and chemical leaching are actively involved in soil remediation, it changes soil properties especially pH, nutrient profile, which in turn reduces soil fertility and it also have small-scale applications. (Nedjimi and Daoud 2009)

Phytoremediation is use of plant to detoxify pollutant from soil and waterbodies. The process of phytoremediation is executed based on five different mechanisms. It depends on hyperaccumulating potential of plant (phytoextraction) and rhizospheric microorganism to stabilize metal (phytostabilization), transfer the pollutant from toxic to nontoxic or less toxic volatile form (phytovolatilization) and degradation of pollutant inside plant body (phytodegradation) (Ghosh and Singh, 2005). As compared to other physical techniques, phytoremediation is well efficient, eco-friendly, low-cost, large-scale application, easy to dispose and most importantly improves soil fertility by releasing organics matters (Saier and Trevors, 2010). Research has already been carried out to enhance phytoremediation process which includes the use of aids like increase soil amendment with biochar (Sugawara *et al.*, 2022), using EDTA (Kamal *et al.*, 2023), different biotechnological approach to produce genetically engineered plant (Bhuiyan *et al.*, 2011). These aids enhance phytoremediation by providing availability of heavy metal, support plant growth and in certain circumstances reducing the toxicity of pollutant. The main objectives of the review is to understand the efficacy of some known hyperaccumulator for its integrated approach in phytoremediation and biomass utilisation through different process. Some plant species such as *Pteris vittata,* different *Brassica* species (*B. juncea, B. napus, B. campestris),* different species of *Asteraceae (Tagetes erecta*, *T.patula, Helianthus annuus*, *Helianthus petiolaris) and Ricinus communis, Jatropha curcas and Cannabis sativa* are successfully involved in extraction of heavy metal like arsenic (As), cadmium (Cd), lead (Pb), nickel (Ni), zinc (Zn), iron (Fe), manganese (Mn) from polluted soil. Following the accumulation of the heavy metals, proper utilization of their plant biomass occurs in the field of biofumigation, biofortification and for bioenergy production (Table 1). This is because the efficiency of phytoremediation not only depend upon by choosing right hyperaccumulators but also on selective utilization of plant biomass to generate bio-product which are used in further processes.

1.1. Criteria of different plant species used in phytoremediation

The plant used in phytoremediation must deal with wide range of pollutants rather than focusing on specific one. Plant should exhibit higher biomass production in above ground part and also possess extensive root system. Moreover, it must be hyperaccumulator, capable of tolerating heavy metal toxicity, easy to cultivate, and show resistance to herbivory (Adesodun *et al.*, 2010).

1.2. Plant species used in phytoremediation

There are approximately 400 plant species belonging to 45 families are reported for hyperaccumulating metal or metalloids (Ghosh and Singh, 2005). Most commonly, hyperaccumulator species belong to families such as Brassicacece, Asteraceae, Euphorbiaceae, Lamiaceae, and Scrophulariceae. Among these, most of the hyperaccumulator are member of family Brassicaceae. The use of the first transgenic plant in phytoremediation of selenium-polluted soil was successfully confirmed by Bauelos *et al.* (2005) and suggested *B. juncea* with overexpressed glutathione synthetase (GS) exhibit significant tolerance for selenium and had more biomass than wild type plants. Similar results were drawn *B. juncea* as a potent species showing highest tolerance to Cd and Pb due to presence of BjGSII (*Brassica junce*ag lutathione synthetase II) and BjPCS1 (*Brassica juncea* phytochelatin synthase 1) induced by over expressed AtATM3 gene. Gurajala *et al.* (2019) investigated the impact of bi-metal (Pb and Cd) contaminated soil with various genotypes of *B. juncea* and confirmed genotypes IM-13, IM-25, IM-65 were more effective for removing Cd followed by IM-24, IM-79, IM-32. Kamal *et al.* (2023) investigated dependent effect of EDTA (Ethylenediaminetetraacetic acid) on *B. juncea* seedlings and concluded highest plant biomass found in 2mM per kg EDTA. Beyond this, both the photosynthetic activity and plant biomass decreased. It was also reported that exogenous supply of EDTA. On contrary, Afshan *et al.* (2015) opined application of citric acid enhanced plant growth, biomass, chlorophyll, significant

Table 1:

Various methods of utilisation of phytotremidiated plant biomass in different families.

Sl No	Plant Family	Biomass utilization process	Outcomes	Reference
1.	Brassicaceae	Biofumigation	Highest antifungal potency exhibited in seed meal inhibiting 61.5% of fungal growth, followed by seed powder, flowering stage, and vegetative stages of fresh plant part in *Brassica juncea.*	Abdallah et al., 2020
2.	Brassicaceae	Biofumigation	White cabbage showed a positive relationship between heavymetal accumulation and the production of bioactive glucosinolates (GLS) production.	Kusznierewicz et al., 2012
4.	Brassicaceae	Biofortification	*Brassica rapa* ESB1 mutant exhibited biofortification for Fe and Cu.	Calvo et al., 2023
5.	Asteraceae	Bioenergy	Oil and bioethanol yield from of *Helianthus annus* is consistent in both agricultural and industrial soil polluted with Zn and Cd.	Paulo et al., 2023
6.	Pteridaceae	Biochar	Phyto remediated biomass of *P. vittata* used as biochar and supply with $FeCl_3$ enhanced the arsenic adsorption by biochar.	Sugawara et al., 2022
7.	Asteraceae	*Biochar*	Biochar obtained from pyrolyzed biomass of *H. annuus* were reutilized as fertilizer.	Zhou et al., 2020
8.	Pteridaceae	Bioenergy	Coupling with ethanol extraction and anaerobic digestion reduced 98% arsenic concentration in *P. vittata* biomass.	Silva et al., 2019
9.	Cannbaceae	Biofortification	*C. sativa*had potential *to* accumulate Selenium in leaves and seed which in turn show biofortifying potential.	Stonehouse et al., 2020

rise in antioxidant enzyme with notable increase in chromium uptake and minimized Cr induced stress in *B. napus.* By analysing bioconcentration factor and morpho-physiological, biochemical analysis, Ali *et al*. (2022) confirmed phytoextraction potential of *Brassica* species in the order of *B. juncea*> *B. napus* > *B. campestris* > *B. rapa* on contaminated soils. Recently Bortoloti and Baron (2022) concluded phytoremediation by *Brassica* species was the most promising approach but it needs further studies to assist utilization of biomass and tolerance.

Biswal *et al.* (2021) investigated efficacy of *Tagetes erecta* and *Tagetes patula* on removal of Cd and Ni from polluted site and observed *T. erecta* and *T. patula* are quite effective at removing Cd and Ni, respectively and as comparison to *T. patula*, *T. erecta* had higher biomass and it more efficiently accumulated heavymetal from contaminated soil. Similarly, Madanan *et al.* (2021) confirmed *T. erecta* showned BCF >1 for Cd and Zn but <1 for Pb which clarify *Tagetes erecta* L. was an efficient hyperaccumulator of Zn and Cd and excluder of Pb. Francis (2018) screened the Phyto-remediating potential of *Helianthus annuus* and observed that the metals are accumulated in various part of plant in the decreasing order of Ni > Pb > Cr > Cd in the plant part. In contrast, Aybar *et al.* (2023) observed *Helianthus annuus* was the good phytoextractor of zinc and lead and stabilized copper in soil found in vicinity of mining area. Sharma and Mathur (2023) suggested *H. annuus* effectively extracted zinc as compare to *T. erecta* and utilized as successful phytoextractor of zinc from polluted soil.

Jatropha curcas effectively accumulated considerable amount of Fe and also extract Pb, Zn, Cu, Cr and Ni with little amount of As, Hg, Sn (Mateos *et al.*, 2019). Kristanti *et al.* (2023) confirmed, *J. curcas* was abled to extract 88.5% of aluminium and showed bioconcentration factor up to 5.62

which indicates potent hyper-accumulator of aluminium. Jain and Tembhurkar (2023) evaluated the potential of remediation and energy yield of *J. curcas, Millettia pinnata* & *H. annuus* in fly ash contaminated soil and observed that *J. curcas* was much efficient phytoremediator. However, *H. annuus* accumulated higher heavymetal but it no longer survived in such condition.

In *Ricinus communis,* the order of metal accumulation was observed as Fe > Zn > Mn >Pb >Cd, which is negatively co-related with concentration of protein and chlorophyll content (Boda *et al.*, 2017). *Ricinus communis* trans located lead in aerial part of plant in the order of shoot > root > leaf and used as an indicator of Pb in contaminated soil (Roychowdhury *et al.*, 2019). Similarly Khan *et al.* (2019), *R. communis* stored considerable amount of heavymetal in aerial parts which reduced heavy metal content of soil.

Pteris vittata L, hadability to withstand at very high concentration of arsenic and stored in its frond. Gaggero *et al.* (2020) observed, *P. vittata* efficiently removed arsenic, while *B. Juncea* showed higher translocation and bioaccumulation for Cadmium and *H. annuus* successfully extracted zinc and cadmium whether as no such result was found in *Zea mays*. These finding also provides the differential accumulation potential of different species for extraction of heavymetal from contaminated sites and recommend the use of plant species in accordance to the target. Kohda *et al.* (2022) observed *Pteris vittata* extracted 2.82 kg arsenic per hector in sub-arctic area which was quite efficient for removal of heavymetal in phytoremediation under such condition. *Pteris vittata* significantly accumulated As and Pb in co-planting but after addition of chitosan, the uptake of Cd and Pb by *Pteris vittata* and *Ricinus communis* increased significantly. However, accumulation of As by *Pteris vittate* was reduced (Yang *et al.*, 2017). Wan *et al.* (2021), explored intercropping system with *Pteris vittata* to enhance the accumulation of arsenic while simultaneously decreasing the concentration of Arsenic in another crop. This study givesnew direction for improving phytoremediation of Arsenic polluted soil with *Pteris vittate* by intercropping methods.

Cannabis sativa accumulates Cu, Cd, and Ni in the leaves due to presence of the stress tolerant genes PLD and GSR in response to heavymetal (Ahmad *et al.*, 2016). Picchi *et al.* (2022) put forward that *C. sativa* showed higher BCF and lower TF for Arsenic as compare to *B. juncea*. However, exogenous supply of phosphate had no role in accumulation of heavymetal but it only increases plant physiological functions. Testa *et al.* (2023) observed that in comparison to Cd and Pb, Ni significantly reduced the biomass of *Cannabis sativa* and further 75 cultivar had more Cd and Pb tolerance *C. Sativa* completed its life cycle until seed bearing phase even in heavily polluted soil which provides new insight for the creation of green energy. The current study provides new perspectives for effectively choosing hyperaccumulator species for long-term phyto-management of a substantially contaminated site using a combined phytoremediation-bioenergy approach for clean-up soil and sustainable development.

2. Integrated approach for utilisation of plant Biomass

2.1. Biofumigation

Generally, the families of order Capparales (Brassicaceae, Capparaceae, Moringaceae) contains more glucosinolates than other families (Euphorbiaceae, Salvodaraceae (Fenwick *et al.*, 1983). Glucosinolates (GSLs) are secondary compounds present in the order Capparales. The breakdown products of GSLs offer a broad range of defence against pathogens (bacterial, fungal, nematodes) and herbivores. Additionally, GSLs play a crucial role in determining the taste and smell of cruciferous vegetables, providing various health benefits. The amount of glucosinolates (in vacuoles) increases when the plant is under attack from a pathogen or experiences stress, and as it comes in contact with myrosinase (in the cytoplasm), it hydrolyzes the thioglucoside bond and releases biologically active isothiocyanates (ITCs), thiocyanates, nitriles, etc. (Andernach *et al.*, 2023). Wu *et al.* (2011) fund the notable decrease in nematode motility upon exposure to isothiocyanates (ITCs) from *Brassica* plants. This observation is further supported by Fourie *et al.* (2016), who highlighted the successful utilization of species such as *B. oleracea*, *B. rapa*, *B. napus*, *B. juncea*, *B. campestris*, *B. nigra*, *E. sativa*, *R. sativus*, etc., for biofumigation. *Brassica* as a cover crop hada number of benefits since it combats soil pathogens, nematode, weeds (Nyczepir *et al.*, 2009) and reduced soil borne disease, as well as enhanc soil fertility and reduce soil erosion (Nyczepir *et al.*, 2009).

According to Kusznierewicz *et al.* (2012), heavy metal accumulation and generation of bioactive glucosinolates (GLS) are positively correlated in cabbage. However, rise in glucosinolates level occurred in a dose-dependent manner which proves excellent bio-fumigation potential of white cabbage. Jakovljeviæ *et al.* (2013) examined effect of Cadmium toxicity with respect to change in concentration of glucosinolates and sulphurcontent and found heavy metals did not significantly alter plant biomass or cause any harmful symptoms. However, the root showed higher Cd accumulation as translocation from root to shoot become saturated. Contrarily, Durenne *et al.* (2018) found increasing Cd dosages result in decrease amount of glucosinolates in

B. napus roots, shoots while increasing the amount of sulphur in other plant parts. Abdallah *et al.* (2020) confirmed biofumigant effect of *B. juncea* and found seed meal (without fat) was more potent by inhibiting 61.5% fungal growth followed by seed powder and flowering stage as compared to vegetative stages. Biofumigation is not only limited to Brassicaceae but also in different families other than it and produce cyanogenic compound which is pathogen repellent in nature. Marigold belongs to Asteraceae, produce α-terthienyl which activated upon ultraviolet range, produce reactive oxygen species which was phytotoxic in nature and also had broad effect against pathogen. Several allelopathic compound like diethynyl, dithioacetylene also possess biocidal property (Dutta *et al.*, 2019). Further study must focus to evaluate the effect of heavymetal concentration and glucosinolates production in plant, the mechanism of activity and the metabolic pathway concerned with glucosinolate production and role of glucosinolate in reduction of phyto-toxicity arise due to heavymetal.

2.2. Biofortification:

High population growth, climate change and deficiency of nutrious food are the major reason of malnutrition in developing countries. Zn and Fe deficiency in human is common in the areas where soil is poor Zn and Fe or have antinutrient which inhibit Fe and Zn absorption (Cakmak, 2008). Several studies of plant genetic engineering along withcrop breeding performed to enhance nutrient like Feins food crops by increased expression of metal binding protein, chelating agent, amino acid (Kumar *et al.*, 2019), increase translocation of Fe and Zn from root to aerialpart and reducing the effect of anti-nutrient phytate (Palmgren *et al.,* 2008). There was great variation among genotype *Brassica* species but it considered as good source of nutrient (Fe and Zn) and substantial amount present in young part of plant during their growth (Gioia *et al.*, 2017). Vegetables belonging to family Brassicaceae have capacity to accumulate selenium (Se) which is an essential micro nutrient and produce seleno compound with numerous health benefits. *B. juncea* successfully extracted selenium from coal mining waste and also accumulates minute amounts of As, Cd, Pb, and Cu (Monei *et al.*, 2021). Although *Brassica rapa* ESB1 mutant did not show biofortification for Selenium however, it minimizes translocation of Cd in the leaves and increase Fe and Cu uptake (Calvo *et al.*, 2023). *Cannabis sativa* also showed biofortifying potential in Se contained agricultural land and accumulated Se in leaves and seed (Stonehouse *et al.*, 2020). Moreover, selective study needs to focus on successful biofortification of essential nutrient in crop from contaminated area.

2.3. Bioenergy

The efficacy of integrated phyto-remediation with bioenergy production assist environment by removing pollutant, better soil quality as well as a viable alternative for sustainable energy source. Bioenergy is a type of renewable energy, generated from biomass of living organism especially plant and generated biomass from phytoremediation process act as indirect source of solar energy. So, it can be used as energy production in different process like Combustion, gasification, and pyrolysis methods. Several plant species have the potential to execute phytoremediation with production of bioethanol, biodiesel, biogas, biochar, charcoal etc. Park *et al.* (2012) successfully extracted heavymetal from the soil and the oil extracted from *B. napus* biomass were used as biodiesel as it contains heavymetal at standard permissible level. Huang *et al.* (2018) observed pyrolyzed temperature increase from 550 °C, there was sharp decrease in heavymetal concentration in plant biomass of *Jatropha curcas* and it was safe for use. Hunce *et al.* (2019) found the seeds of *H. annuus* produced highest biogas in anaerobic digestion which further satisfy sustainable green energy from hazardous Phyto-biomass.

Cannabis sativa was a potent hyper accumulator of heavymetal in polluted site with characteristic high biomass which utilized for production of biogas, bioethanol, biodiesel, bio methanol (Kumar *et al.*, 2017). Todde *et al.* (2022) opined on *C. sativa*that anaerobic digestion and incineration of that biomass are used for most sustainable production of energy by reducing the production of CO_2 into the atmosphere. By combining ethanol extraction and anaerobic digestion, Silva *et al.* (2019) created a unique approach for decreasing arsenic in *Pteris vittata* biomass by 98% and remaining Arsenic in *Pteris vittata* biomass consider as safe material based on standard toxicity limit. The combination of integrated phyto-remediation with bioenergy production offers a novel perspective on the sustainable production of energy, presenting an alternative method for utilizing plant biomass.

2.4. Biochar

Biochar is the carbonized product produced from thermal decomposition of plant biomass anaerobically and used as soil conditioner, carbon dioxide sequestration, and in energy production. Nevertheless, it is crucial to regulate the quality of biochar, especially when it produced from waste source (Lehmann and Joseph, 2015). Zhou *et al.* (2020) provided an innovative approach to reduce the bio hazardous phytobiomass of *H. annuus.* From pyrolyzed biomass, metals were separated by acid extraction followed by alkaline precipitation method and the biochar obtained

from this were reutilized as fertilizer. Lee *et al.* (2021) reconfirmed same result in *H. annuus* where production of hydrochar resulted in heavy metal content within a safe range. Sugawara *et al.* (2022) observed phyto-remediated biomass of *Pteris vittate* when transformed into biochar and treated with $FeCl_3$, indicate rapid raised in arsenic adsorption. Although biochar production required high temperature however, more study would reveal the sustainable production of biochar from phytoextracted biomass and proper utilization in soil amendment.

3. Summary and future perspectives

Schematic diagram (Figure 1) reflects the heavy metal sources and their deposition in soil. The concentrated heavy-metals are minimized by using green technology called phytoremediation. In this the plant extract heavy metal from soil (phytoextraction), converts to less toxic form (phytovolatilization), and stabilize heavymetal in soil (phytostabilization). Later, the biomass was used to produce biochar, which assisted in phytoremediation to adsorb heavy metals. Furthermore, it also utilized to produce bioenergy (bioethanol, biofuel). Biomass also utilised as biocides and enrich nutrient in crop by bio fumigation and biofortification respectively. Further study must be focused on metabolic pathway, enzymes, gene expression, fate of heavymetal inside plant tissue, which provide tolerance to plant species and efficiently extract heavymetal from polluted sites. Further research is required to comprehend the production of glucosinolates or cyanogenic glycosides induced by heavy metals. This can lead to the development of natural biocides with fewer environmental side effects.

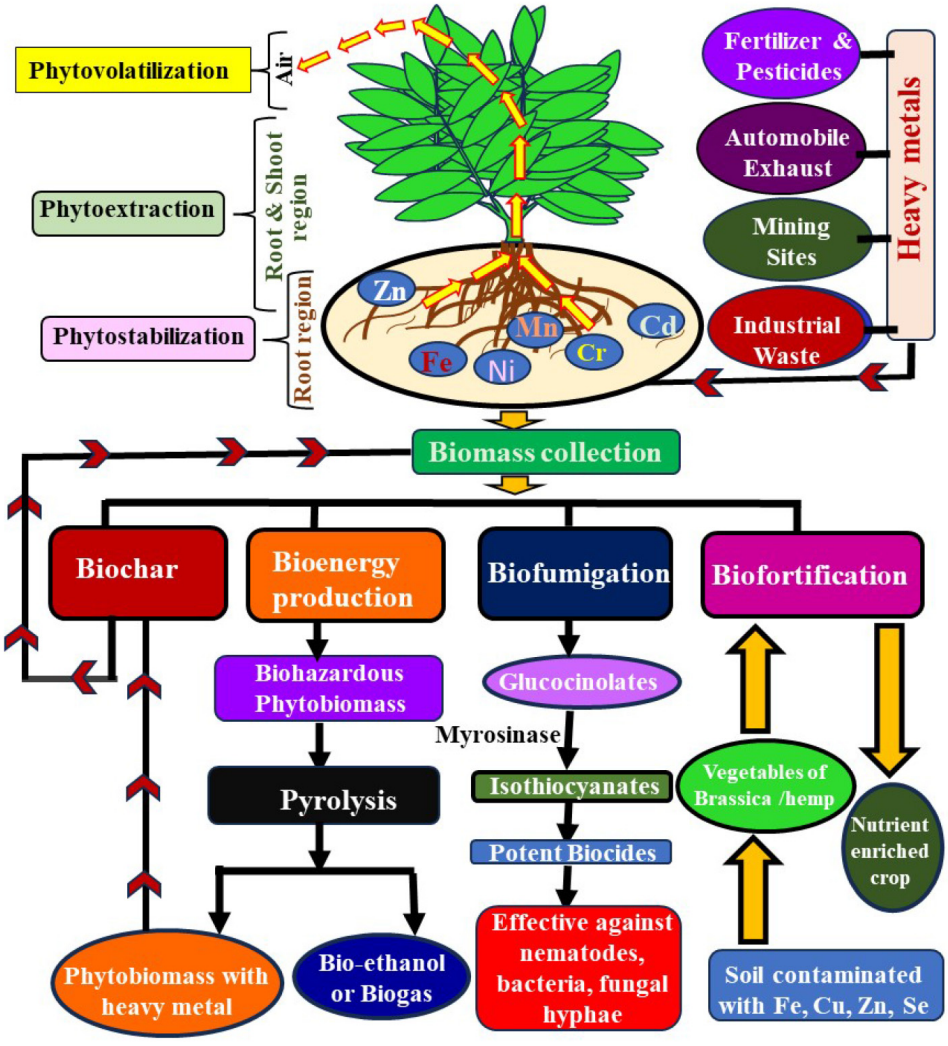

Figure 1: Schematic diagram showing integrated approaches towards efficient phytoremediationby using green technology. It summarises various sources of heavy metals, their deposition in soil, the ways to minimize though phytoextraction, phytovolatilization and phytostabilization. In the next step, the biomass was used to produce biocharto adsorb heavy metals, to produce bioenergy (bioethanol, biofuel), biocides for better enrich nutrient in crop by bio fumigation and biofortification.

4. Conclusion

In the current scenario, the problem associated with heavymetal is a persistent issue. Phytoremediation is emerging solar energy driven technique which utilizes the plants for removal of heavy metal (inorganic and organic form) without disturbing the physiological characteristic of soil. Phytoremediation of metals may be enhanced by using aids like soil amendment with biochar, EDTA, citric acid, rhizospheric association between endophytic bacteria, other biotechnological approaches to produce genetically engineered plant. Efficient phytoremediation of heavymetals requires the proper utilization of plant biomass using hyperaccumulator. Future research in this area would provide new insight for sustainable development of ecosystemas well as the toxic heavy metal can successfully be extracted and efficiently employed for generation of bioproduct using the integrated approaches.

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Formulation and Process Development of Biofuel and other products from Mahua (*Madhuca latifolia* L.)

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ABSTRACT

The genus *Madhuca*, belonging to the family of Sapotaceae, is a multifunctional tree and is considered a boon by the tribal's who are forest dwellers and keenly conserve this tree. Mahua flower, seed, fruits and their products are used as a food, fodder and are a non-conventional ingredient in carp feedas well as used as an exchanger in tribal and rural areas. Mahua seeds are rich in edible oil so they have economic importance. Mahua seed oil proved to be cheap and suitable substrate for the production of biofuel. The bioethanol production from Mahua flowers could be increased by developing the traditional methodology, as it is expected to benefit the people of tribal areas in India as well as the bioenergy demand in world in the long run. Flowers have been reported to have various bioactivities and ethno-medicinal uses. Mahua flowers are known to play an immense role in the preparation of valued added food products (biscuit, cake, laddu, candy, bar, jam jelly, sauces etc).

1. Introduction

Mahua (*Madhuca* sp.,) trees are widely distributed in the forests of the Australian and Asian continents (Gupta *et al*., 2012). It is a major tree in the tropical mixed deciduous forests of West Bengal, Bihar, Odisha, Madhya Pradesh, Punjab and Uttar Pradesh as well as the sub-mountainous region of the Himalayas in India (Gupta *et al.,* 2012; Sinha *et al*., 2017) since it is adapted to arid situations and produced in excess of a million tonne annually (Gupta *et al*., 2012; Bakhara *et al*., 2016). In a report, Bakhara *et al*. (2016) reported that Mahua has been widely planted in the Deccan Peninsula and Northern India's plains, and it is usually propagated by seeds (Gupta *et al*., 2012; Bakhara *et al*., 2016). Mahua flower is one of the most important Non-timber forest products (NTFP), playing a major role in the tribal economy (Bakhara *et al*., 2016). Its many parts have been used to make food, fuel, fertiliser, cattle feed and oil (Ramadan *et al*., 2016; Behera & Ray, 2019).

In many states, cooperative corporations, purchase flowers at a minimum support price to protect collectors from the middleman's exploitation. However, the majority of the flowers degrade/get decomposed in the government go downs because to a lack of adequate post-harvest processing technology (Bakhara *et al*., 2016). Value added food products from Mahua have been modified or enhanced to have a higher market value and/or a longer shelf life. Different marketable value-added food products like dried flower, candied flower, glazed flower, Mahua bar, Ready-to-Serve beverages (RTS), squash, jam, laddu and cake were prepared and the products were well appreciated by the consumers (Bakhara *et al*., 2016). In this project, specified marketable products such as Mahua powder, candy/toffee/ lollipop, laddu and biscuits/cake, soft drink/cold drinks added ethnomedicinal values (antidiabetic, anticarcinogenic, anti-inflammatory, antimicrobial, etc.) has been prepared for the consumers (Badukale *et al*., 2021).

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Mahua has helped to improve the socioeconomic conditions and rural livelihoods of these communities (Behera & Ray, 2019). In addition, it has been used to better understand the relationships between various socioeconomic factors (location, wealth status, gender, education, and seasonality) that affect the degree of dependence on Mahua, in various districts and agro ecological zones of India.

2. Opportunity

Economic security accompanied by food security can be provided to the tribal by exploring the opportunities of Mahua plant (Nayak & Sahoo, 2020). However, various medicinal and functional properties have been accorded to the various parts of the tree. According to several researches, there is a need for strategy for the harvesting of flowers with appropriate value addition; this is a serious attempt to provide information on the various food products and edible uses of flower, fruit and seed of Mahua tree targeting it to provide prominent raw materials to the industry (Nayak & Sahoo, 2020). Mahua flowers can be seen as sugar replacers and formulated to form various functional foods. Mahua fruit can be minimally processed to be available as a vegetable source. Mahua seed oil provides array of applications as butter replacer or blending oil. There is a need to boost Mahua in our economy as the tree is a boon for the tribal across Indian forests (Behera & Ray, 2019).

3. Process Development of Biofuel

The quest for new and inexpensive carbohydrates sources for the development of bioethanol and biodiesel is gaining traction around the world. Mahua flowers and seed oil are an alternative forest biomass to develop bio-refinery production (Banerjee and Samanta, 2018; Bai *et al*., 2021).

3.1. Bioethanol from Mahua Flower

The recent studies have demonstrated that Mahua flowers can serve as a renewable feedstock for commercial bio-ethanol production (Swain *et al*., 2007; Mohanty *et al*., 2009; Behera *et al*., 2010a; 2010b; 2012; Behera *et al*., 2016). Mahua flowers are a natural, non-food quality, low-cost carbohydrate substrate found in non-agricultural environments or forest ecosystem that can be used to make bioethanol instead of food-grade sugar/starchy crops like maize and sugarcane (Behera *et al*., 2016). Mohanty *et al*. (2009), *Saccharomyces cerevisiae* used solid state fermentation (SsF) to produce ethanol from Mahula flowers. The maximum ethanol concentration (225.0 g/Kg flower) was obtained after 72 hours of fermentation and found to be optimal at ideal parameters (70% moisture, pH 6.0, and 30°C). Batch fermentation of Mahula flowers was carried out by Behera *et al*. (2010) using immobilised cells (in agar agar and calcium alginate) and free cells of *S. cerevisiae*. Using immobilised (in agar agar and calcium alginate) and free cells, the ethanol yields were found 151.2, 154.5, and 149.1 g/Kg flowers, respectively. Furthermore, the immobilised cells remained physiologically active for at least three ethanol fermentation cycles without reducing productivity. In an another study, Behera *et al*. (2010a) reported the bioethanol production from mahula flowers using immobilised *S. cerevisiae* and *Z. mobilis* in calcium alginate beads. Using immobilised (calcium alginate) *Z. mobilis* and *S. cerevisiae* cells the ethanol yields were reported to be 154.5 and 134.55 g/Kg flowers, respectively. Gedela *et al*. (2016) described a fermentation process using *S. cerevisiae* to produce bioethanol from Mahua flowers. The presence of bioethanol and the percentage of bioethanol were verified using a spectrophotometer (204–240 nm) and an alcohol metre, respectively. The results revealed that 1000 mL of acidic fermented media (pH-4, 5 and 5.7) contained 170.03, 142.3, and 27.7 mL of bioethanol in test-1 (*S. cerevisiae* + Mahua flowers + media), test -2 (Mahua flowers + media), and control (media) respectively. The development of first generation biofuels (bioethanol) from Mahua flowers enriched with high amounts of fermentable sugars was reported by Banerjee and Samanta (2018). For the development of bioethanol from Mahua flower extract, the fermentation method (batch and fed-batch) used yeast strain *S. cerevisiae*-3078 culture. From fresh and 6-month-stored Mahua flowers, the maximum yields of ethanol (33 °C and pH 5.7) after 14 days were found to be 18 and 15% (using batch) and 22 and 16% (using fed-batch fermentation), respectively. Agrawal *et al*. (2019) used a newly isolated strain of *Pichia kudriavzevii* from milk whey to produce bioethanol from Mahua flowers. Using Mahua flowers as a substrate, the isolated yeast was tested for fermentation capacity using a carbohydrate fermentation test. Optimizing operational parameters (fermentation time of 48 hours, temperature of 25°C, and pH of 5.0) resulted in a maximum ethanol yield of 371 g/Kg from the flowers.

3.2. Biodiesel from Mahua seed Oil

Biodiesel (fatty acid alkyl ester) has the following advantages to meet the future energy demands; it is biodegradable, environmentally safe, non-toxic, and sustainable (Puhan *et al*., 2005; Kumar *et al*., 2018). Biodiesel is made from animal fats, vegetable oils, recycled cooking greases, and waste plastics all over the world. Globally, edible oils such as sunflower, peanut, canola, palm, coconut, and other oils account for 95% of biodiesel output. Owing to the impending scarcity of food crops, these biodiesel productions are not advantageous from edible oils (Atabani *et al*., 2012; Ansari *et al*., 2022). It should be obvious to

researchers that they look for advantageous biodiesel sources and concentrate on feedstocks that do not include food crops. Mahua oil is used to keep the cost of vehicle fuel low and stable. As a result, non-edible Mahua oil was chosen as one of the key alternatives to diesel oil (Karmakar *et al*., 2010; Singh and Singh, 2010; Ansari *et al*., 2022).

Transesterification can be used to make biodiesel from any Mahua seed oil. Ghadge and Raheman (2005) developed a method for producing biodiesel from Mahua oil (*M. indica*), which contains a high amount of free fatty acids (FFA). The pre-treatment process was reduced the high FFA content of Mahua oil with a methanol-to-oil ratio (0.30-0.35 v/v) in the presence of H_2SO_4 (1%, v/v) as an acid catalyst. Mahua biodiesel fuel properties were found to be similar to those of diesel, and it met both American and European standards. Kumar *et al*. (2018) reported that the Mahua seed oil was trans-esterified with methanol using acid and alkaline catalyst process to obtain Mahua methyl ester (MME). MME was examined for its chemical composition and physical properties. The performance, combustion, and emission of a computerised single cylinder CI engine fuelled with diesel and B20 (20% vol. of MME biodiesel + 80% vol. diesel) were investigated in experimental tests. The B20 fuel out performed the baseline diesel in terms of efficiency at lower partial loads, combustion, and emissions. The combination of B20 with smaller orifice NHD has shown appreciable results in performance, combustion and emissions. But, the only drawback was NO_x is found to be increased.

Since Mahua oil contains a high amount of FFA, it is difficult to turn it into biodiesel using a chemical catalyst. Kumari *et al*. (2007) used a commercial preparation of lipasefrom *Pseudomonas cepacia* as a catalyst for transforming Mahua oil into ethyl esters. Biocatalyst formulations such as cross-linked enzyme aggregates (CLEAs) and protein-coated micro catalysts (PCMCs) produced the best performance. Using 50 mg of lipase, free enzyme powder converted to 98% in 6 hours, CLEAs converted to 92% in 2.5 hours, and PCMCs converted to 99% in 2.5 hours after process optimization. It was also reported that upon further optimization with PCMCs, a more economical and efficient process design would be possible.

Use of non-edible Mahua oil (MO) as fuel in agricultural diesel engine will improve rural economy, sustainability and increase the environmental benefits (Kapilan *et al*., 2009). They reported that the flash point, density and viscosity of the MO are higher than the diesel, but it has lower calorific value. Mixing diesel with the MO reduces the viscosity of the blends of MO and diesel. From the analysis, it was claimed that the MO can be partially substituted for diesel oil in the diesel engine, without making any modification in the hardware of the engine.

The effect of injection opening pressure (IOP) for 20% blend (B20) of Mahua oil methyl ester (MOME) and 22.5 litres per minute (lpm) of hydrogen dual fuel mode was investigated by Syed *et al*. (2017). A single cylinder, four stroke, direct injection (DI) diesel engine (3.3 Kw, 1500 rpm) was studied for its efficiency, combustion, and emission characteristics. The maximum brake thermal efficiency, minimum brake specific fuel combustion, and lowest HC, CO, and smoke emissions with increased NOx concentrations were obtained at IOP of 250 bar for B20-hydrogen dual fuel mode (Syed *et al*., 2017).

Pradhan *et al*. (2017) investigated the processing, characterization, and potential applications of Mahua oilseed bio-oil. The process of pyrolysis was carried out between 450°C and 600°C in a semi-batch style reactor for the development of Mahua pyrolysis oil (MPO). At an optimum temperature of 525 °C, the MPO yield was found to be about 50%. The MPO was also characterized for its suitability as an alternative fuel for internal combustion engines.

The efficiency and emission characteristics of a direct injection (DI) diesel engine with cerium oxide nanoparticles additives in diesel and biodiesel blends were studied by Seela *et al*. (2019). Transesterification was used to make Mahua methyl ester, which was then mixed with diesel. According to the findings, the thermal efficiency of B20 + 100 ppm cerium oxide brakes increased by 1.8, with a 1% reduction in real fuel consumption. Hydrogen and carbon monoxide emissions were lower than with diesel fuel.

Vijay Kumar *et al*. (2019) investigated two methods of transesterification to produce Mahua methyl ester (MME) from raw Mahua seed oil. The physical properties of the samples were compared to the ASTM D-6751 requirements. In an unmodified diesel engine, the obtained MME and its blends of B20, B40, B60, and B80 were investigated. It was observed that brake-specific fuel consumption and thermal efficiency are slightly improved (B20 and B40) at part-load conditions and approach diesel at full-load conditions based on the results of the performance. From combustion analysis, it was found a shorter ignition delay for biodiesel and its blends compared with the diesel fuel. Both of the fuel blends reduced carbon dioxide, hydrocarbons, and smoke opacity emissions, but at high temperatures, they undergo an endothermic reaction that produces various nitrogen oxides.

Bai *et al*. (2021) investigated the production of methyl ester from Mahua oil through esterification and transesterification. The methyl ester of Mahua oil was

prepared under a variety of conditions (methanol to oil molar ratio, concentration of catalyst, effect of temperature, and reaction time). The results shown that 6:1 liquor to oil molar proportion with 1% KOH impetus, 60 °C response temperature and time 120 min gives 92% ideal yield are the optimum conditions for esterification. The ASTM standards were used to compare the different fuel properties of Mahua oil methyl ester. To recognise and confirm the existence of fatty acids methyl esters, GCMS and FTIR analysis were used. The Mahua oil was found to be a possible raw material for the methyl ester. It is affordable and could become a viable renewable fuel in the near future.

The reduction of thermal efficiency by Mahua mixed biodiesel has major challenge (Ansari *et al*., 2022). They reported the performance and emission characteristic of Mahua as blended biodiesel. The natural diesel blended with the proportion of starting increasing from (5 to 20 %) of Mahua seeds oil and starting reduction from (95 to 80 %) of natural diesel oil. The diesel engine was operated on the basis of different Mahua seeds oil quantity with natural diesel, and it was observed the highest thermal efficiency of blended biodiesel were at (20 %:80 %) of Mahua seeds oil and natural diesel oil respectively.

4. Process Development of value - added food products

A good source of value-added products can be made from the Mahua flowers, fruits and seed oil (Dwivedi *et al*., 2022).

4.1. Nutritional Value

Sugar profiling of Mahua flower revealed that it is a rich source of both reducing sugar (48–75%) and non-reducing sugars (3–18%) such as inositol, sorbitol, dextrose, fructose, sucrose, raffinose, and maltose (Singh *et al*., 2020; Dwivedi *et al*., 2022). The nitrogen content of the flower varies from 0.65-1.1% being apparently higher in the younger than in the flowers varies from 4.4-7%. Similarly, the fat content of dry flowers varies from 0.09-1.3%. Calcium, phosphorus, iron, potassium, sodium, and magnesium are among the several minerals found in the flowers (Pinakin *et al*., 2020). Adequate quantity of other vitamins, including as thiamine, riboflavin, niacin, folic acid, and ascorbic acid, are also found in the flowers. Enzymes like amylase, maltase, invertase, catalase and oxide was detected due to the high rate of degradation of the vitamin c content in the storage experiments (Bakhara *et al*., 2016; Singh *et al*., 2020; Dwivedi *et al*., 2022).

4.2. Food Value

The juice extracted from the Mahua flower can be used as a natural sweetener in a variety of foods, including biscuits, cookies, cakes, jam, jelly, juice, and squash (Figure-1). Furthermore, Mahua flowers are commonly used by locals to make alcohol and are processed by many locals for use as a food source in the winter (Singh *et al*., 2020; Dwivedi *et al*., 2022). There exists a tremendous scope for development and value addition of Mahua flower for economic development of the tribal community (Dwivedi *et al*., 2022).

Figure 1. Value added food products from Mahua flowers

5. Other developed Products

In addition, to value added food products, the other marketable value products like Mahua oil cake has been prepared from Mahua flower which has been well appreciated by the consumers. Mahua deoiled cake (MDC), a by-product obtained during extraction of oil from Mahua seed is a plentiful resource of protein, nitrogen, lipids and carbohydrate etc. (Gupta *et al*., 2012). MDC is widely used in several industrial applications such as biogas production, biotechnological application for mushroom cultivation, lipase production or other biochemical production (Ramachandran *et al*., 2007; Biswal *et al*., 2019). MDC is also used as substitute for protein hydrolysates during the treatment of protein malnutrition for animals (fish, cattle etc.) (Biswal *et al*., 2019).

5.1. Mahua Oil Cake in Aquaculture

Feed is the most important factor that significantly affects aquaculture production and profitability. Aquaculture's net revenue would grow if feed costs is reduced and techniques that promote nutrient efficiency has implemented (Rath *et al*., 2017). Mahua oil cake (MOC) is one of the non-conventional ingredients which are generally used as manure for horticultural crops. MOC is rich in protein (24%) and energy (19.0 KJ/g) with high levels of fatty acids comprising saturates (45%), monoenes (42%) and polyunsaturated fatty acids (PUFA, n-6) (7%) (Rath *et al*., 2017). They reported that MOC added to the diet of rohu (*Labeorohita*) fingerlings at a rate of 300 g/Kg and caused without having any negative effects on growth, survival, or nutrient uptake. However, Mahua oil cake can also be used as cheap source of nutrient for fish replacing conventional plant ingredient by solid state fermentation (SsF) (Gupta *et al*., 2018; Das *et al*., 2022). Gupta *et al*. (2018) reported the de-oiled Mahua seed cakes, via solid state fermentation (SsF), producing proteases and cellulases as value added products. The SsF provided a suitable method to degrade their anti-nutritional factors, along with producing enzymes as value added products. The detoxified cakes can be further employed as quality manure and/or animal feed with improved digestibility.

The SsF of Mahua oil cake was mixed strain of microbes (*Sachharomyces cerevisiae* and *Bacillus subtilis*) which improved the quality of Mahua oil cake was reported by Das *et al*. (2022). The increase in crude protein, decrease in crude fibre and decrease in anti-nutritional factors like total tannin and saponin was claimed in SsF mixed Mahua oil cake, without interfering growth, digestibility and immunological parameters in rohu (*L. rohita*) fingerlings (Das *et al*., 2022). Mahua oil cake act as a major piscicide and the extract possess saponins, which are poisonous and are used for fish killing (Rajput & Gaur, 2015). More recently, Saha and Saha (2022) reported the use of Mahua oil cake (MOC), as a piscicide in pre-stocking pond preparation for composite fish culture.

5.2. Essential oil from Mahua flowers

The corolla of flower is fleshy, and cream colored having characteristics odor and source of essential oil (Suryawanshi & Mokat, 2019). Suryawanshi & Mokat (2019) isolated the essential oil Mahua flowers and identified its chemical compositions. The chemical composition of isolated essential oil was investigated by using gas chromatography coupled with mass spectrometry (GC/MS). The isolated essential oil was found to be abundant in sesquiterpenes and could be a good source/precursors of Fernesol and Fernesene. Further studies are needed to investigate the biological activities of this essential oil.

5.3. Mahua flowers as Carbon source

5.3.1.Bacterial polyhydroxyalkanoate (PHA)

Mahua flowers are an organic substrate that are about 60% sugar and also include organic acids that are necessary for the synthesis of copolymers (Anil Kumar *et al*., 2007). Anil Kumar *et al*. (2007) reported that the utilization of Mahua flowers, as a carbon source for bacterial (*Bacillus* sp-256) fermentation intended to produce polyhydroxyalkanoate (PHA). In PHA production medium, nearly 50% of the cost is due to carbon sources such as sugar and organic acids. This can be economized by using industrial by products or natural substrates. Mahua flower can be viewed as a less expensive source of carbon for the production of PHA copolymers, second only to molasses.

5.3.2. Synthesis of Fumaric acid

6. The use of commercial carbon sources, which are expensive, is the main cost factor affecting the manufacture of fumaric acid. By exploiting naturally occurring, less expensive substrates rich in carbon, production costs can be decreased (Singh *et al*., 2021). Singh *et al*. (2021) reported the utilization of Mahua flowers as a cheaper carbon source and low cost production medium for cost-effective fumaric acid production using *Rhizopus oryzae*. The fumaric acid production using Mahua (24.1 g/L) was comparable to the fumaric acid obtained from glucose.The result claimed that the feasibility of low-cost natural substrate for cost-effective fumaric acid production using *R. oryzae*.

7. Scope for Economic Development

Mahua-based products have given numerous opportunities rural economies and are regarded as an

important source of income for tribal people, particularly in India. Mahua-based products are a major source of basic necessities for a sizable portion of India's rural population. A large number of rural populations are dependent on the Mahua based value added food products for fulfilment of their basic needs (Nayak & Sahoo, 2020). From an economic stand point, the oil yield is always the main consideration when determining if a plant is suitable for industrial use. The seeds of Mahua are among those that are underutilised for oil production, as is typical with many other seeds from tropical fruits. This can be because there is not enough technical knowledge about its characteristics and possible applications (Ramadan *et al*., 2016).

8. Conclusion and Future Prospective

The Mahua plant plays a crucial role in the tribes' way of life and serves as one of their most important and main sources of food. The Mahua products, however, are not widely marketed or used. It was observed that tribal dwellers are selling the Mahua flowers and seeds to local markets or intermediaries due to lack of post-harvest management and not having the potential for restoration and lack of access to market. The price of Mahua flowers and their products is higher in metropolitan and global markets than in the local markets. However, the most advantages from Mahua based products for local communities can be achieved by using participatory community-based development initiatives.Taking propercare of the flowers in the post-harvest phase tomaintain the quality and preparing different value added food products from it in small scale couldcontribute for improving the economy of tribalpeople.

Expanding the market for Mahua flowers, diversifying the uses of Mahua flowers and seeds, building vital infrastructures for Mahua flower processing, developing the skills of Mahua flower and seed collectors, and planting high-yielding Mahua trees are some suggested actions that could be taken to improve the rural economy, particularly for those who live in the forest.

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Influence of environmental factors on turmeric (*Curcuma longa* L.): Novel strategies to augment curcuminoid production

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ABSTRACT

Turmeric (*Curcuma longa* L.) belonging to the family Zingiberaceae is known worldwide for its multipurpose use; in medicine, cosmetics, food flavour and textile industries. Several value-added products obtained from turmeric includes the turmeric powder, essential oil, oleoresin and curcuminoids. Curcuminoids are phenylpropanoid derivatives and belong to diarylheptanoid class of secondary metabolites. The curcuminoids of turmeric include curcumin, demethoxycurcumin and bis-demethoxycurcumin. Curcuminis most sought after worldwide due to its tremendous medicinal importance.The curcumin content varies among turmeric cultivars. Environmental factors and growing locations influence variation in transcript levels of key curcuminoid biosynthesis pathway genes in turmeric cultivars which ultimately lead to the alteration in curcuminoid content. In this context, proper identification of elite turmeric cultivars by use of molecular and biochemical markers is necessary. Similarly, high yielding turmeric cultivars which are being produced must be subjected to agroclimatic filed trials prior to their release, which will largely benefit the turmeric industry. Metabolic engineering of curcuminoid biosynthesis pathway in *E. coli* and *S. cerevisiae* further provide a promising approach to produce these compounds in greater scale. The artificial neural network model will also greatly help to increase the yield of curcumin.

1. Introduction

Plant secondary metabolites (PSMs) are the compounds that are not essential for plant metabolic processes but are vital for a plant to interact with the environment. The plants communicate with external stimuli by the help of these compounds. These compounds are accumulated in the plant through various biochemical processes (Pavarini *et al*., 2012). PSMs are synthesized in environmental stress conditions and aid the plants to tackle the stress. The developmental as well as physiological stage of a plant is crucial for secondary metabolite production. Besides, they protect the plants from harmful UV light and help in seed dispersal.

Turmeric (*Curcuma longa* L.) a member of family Zingiberaceae has been in use in traditional medicine and as edible dye for many centuries. Severalvalue-added products can be obtained from turmeric which includes the turmeric powder, essential oil (leaf and rhizome), oleoresin and curcuminoids. Curcuminoids of turmeric comprise the compounds curcumin, demethoxycurcumin and bisdemethoxycurcumin. European Pharmacopoeia considers turmeric rhizome as an official medicinal product. Because of high medicinal importance, the demand for curcumin is increasing and thus a major growth in curcumin market is expected in future. India enjoys a monopoly of turmeric supply in international market (Angles *et al*., 2011). Turmeric essential oil is extracted by steam or hydro distillation procedures from both leaves and rhizomes. The characteristic turmeric aroma is due to the compound Tumer one which is the major compound of the turmeric essential oil (Sacchetti *et al*., 2005). Turmeric oil possesses antioxidant paracitidal and larvicidal properties (Ali *et al*., 2015).

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2. Effect of environmental factors on turmeric phytoconstituents

Plants often encounter various abiotic stresses such as drought, salinity, light, and temperature. These abiotic stresses remarkably cause alterations in plant growth and metabolism There are reports suggesting that 50-70% of crop yield losses are due to these environmental stresses. For sustaining these stress conditions plants respond by altering their metabolic pathways and undergo genetic modifications (Dos reis *et al*., 2012). The environmental and agroclimatic factors largely influence the secondary metabolite composition in turmeric and are responsible for the variation in curcumin content among turmeric cultivars (Anandaraj *et al*., 2014). Garg *et al.* (1999) showed that, variation also existed in the essential oil and curcumin content of *C. longa* grown in North Indian Plains. They collected 27 turmeric accessions and found that the essential oil percentage varied from 0.16% to 1.94% while the curcumin content ranged from 0.61%-1.45%. According to Lee *et al*. (2014) the curcuminoids content are affected both by the geographical location as well species. Their results revealed significant differences in the curcumin content of same species grown at two different regions. Light is an important factor which affects the quality and productivity of turmeric (Srikrishnah and Sutharsan, 2015). UV-B radiation induces the synthesis of L- phenylalanine ammonia-lyase (*PAL*) and peroxidase (*POD*) enzymes (Lavola *et al*., 2000). *PAL* is an important enzyme for the curcuminoid synthesis as the starter substrates involved in curcuminoid biosynthesis are synthesized from phenylalanine (Katsuyama *et al*., 2009). The availability of light plays an important role in the production of phenolics and terpenes (Ingersoll *et al*., 2010). Padmapriya *et al*. (2016) reported an increase in curcumin and total phenol content in turmeric plants grown under shade conditons (25-30%). Hossainand Ishimine (2005) cultivated turmeric under three different soil types (dark-red, grey and red soil) and found that dark red soil favoured the turmeric resulting in higher curcumin content than other soil types. Furthermore, high levels of any one nutrient solely increase the curcumin content (Srivastava *et al*., 2013). The combination of all the organic and inorganic factors is essential for higher curcumin content.

3. Effect of environmental factors on curcuminoid gene expression

A wide range of abiotic and biotic factors regulates the secondary metabolite biosynthesis in plants (Liu *et al*., 2015). Phenylpropanoids are one of the most important groups of secondary metabolites and key enzyme involved in their synthesis is *PAL* enzyme. There are reports of *PAL* regulation at transcriptional level by abiotic factors and pathogens (Khan *et al*., 2004). Curcumin is a derivative of phenylpropanoid and belongs to diarylheptanoid class of secondary metabolites (Roughley and Whiting, 1973). These diarylheptanoids are synthesized by Type III polyketide synthases (PKSs) (Schroder, 1997). Initially it was proposed that the curcuminoids are synthesized by two Type III (PKSs) which include diketide-CoA synthase (*DCS*) and curcumin synthase 1 (*CURS1*) enzymes. *DCS* is involved in the catalytic conversion of feruloyl-CoA and malonyl-CoA to feruloyldiketide-CoA (Kita *et al*., 2008) while *CURSl* catalyzes the conversion of feruloyldiketide-CoA to curcumin. *DCS* and *CURS1* enzymes are also involved in the synthesis of bisdemethoxycurcumin (Katsuyama *et al*., 2009). Subsequently Katsuyama *et al*. (2009) also identified two other type III polyketide synthases (PKSs) that are involved in curcumin biosynthesis viz. *CURS2* and *CURS3*. They have reported that feruloyl-CoA acts as a starter substrate for *CURS2* and *p*-coumaroyl-CoA and feruloyl-CoA acts as a starter substrate for *CURS3*. *CURS2* plays an important role in curcumin and bisdemethoxycurcumin while the *CURS3* participates in synthesis of all the three curcuminoids (Katsuyama *et al*., 2009). The curcuminoid biosynthesis pathway can be divided into upstream and downstream categories. The upstream genes comprise of *PAL, C4H, 4CL, HCT, C3H* and *OMT* while the downstream genes include the *DCS, CURS1, CURS2* and *CURS3* (Deepa *et al*., 2017) (Fig 1).

Elizabeth *et al*. (2011) have reported the variation in curcumin content within turmeric cultivars. They opined difference in the expression levels of genes encoding key enzymes in curcuminoid biosynthesis pathway leads to the variation in curcumin content (Katsuyama *et al*., 2009). Lovdal *et al*. (2010) studied the effect of environmental parameters on the different flavonoid pathway genes in tomato. They used different combinations of nitrogen and light. Flavanone 3-hydroxylase (*F3H*), flavonol synthase (*FLS*) and chalcone synthase (*CHS2*) gene expression was higher at low nitrogen and high light intensity. Similarly, the transcript levels of *PAL5* and *PAL6* also peaked at low nitrogen and high light intensity which suggests that nitrogen is an important factor contributing to flavonoid pathway. Resmi and Soniya (2012) characterized two new Type III polyketide synthase (PKSs), CIPKS9 and CIPKS10. The sequence of CIPKS9 was similar with chalcone synthase and CIPKS10 showed sequence similarity with curcuminoid synthase. They have analyzed the tissue specific expression of these genes and found that the *CIPKS9* transcript accumulation was higher in shoot and rhizome and less in leaves while the *CIPKS10* expression was higher in leaf and low in rhizome. Sheeja *et al*. (2015) studied the *CURS* gene expression in rhizomes of two turmeric cultivars, one

contained high curcumin content (*C. longa*) and other contained low curcumin content (*C. aromatica*). They have reported the up-regulation of curcuminoid pathway genes in *C. longa* and identified two novel PKSs that showed higher transcript accumulation in *C. longa* as compared to *C. aromatica.*

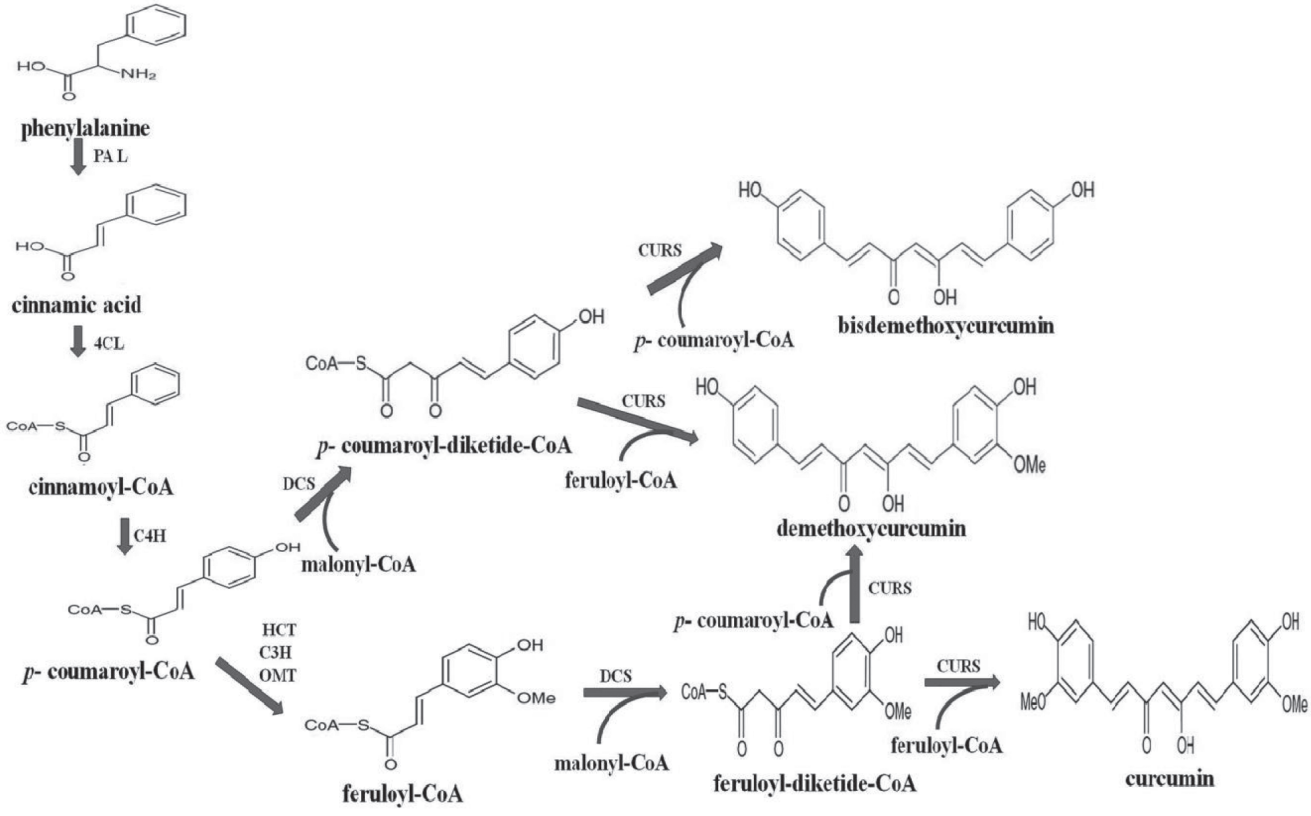
Li *et al*. (2015) studied the expression of curcuminoid biosynthetic pathway genes in four species (two wild and two cultivated). They have found that the expression of *CURS1* and *CURS2* genes was high in cultivated types while the *DCS* gene expression was comparatively low. But in wild types, *DCS* was highly expressed due to greater amount of p-coumaroyldiketide-CoA and they suggested that the difference in the expression could be due to difference in the availability of substrate concentration. According to Deepa *et al*. (2017) curcumin accumulation often exhibit spatio-temporal and environmental variation They studied the effect of environment on the *PKS* (*DCS, CURS1, CURS2* and *CURS3*) genes by selecting two turmeric genotypes with high curcumin (IISR Prathiba) and low curcumin content (Accession 449). The genotypes were planted at two different regions (Kozhikode and Coimbatore) and have found that the expression pattern was similar in both the genotypes where the plants at Kozhikode showed the highest expression and the plants at Coimbatore had the least expression, which clearly shows the impact of environmental factors and growing location on the curcuminoid biosynthetic genes.

Phenylalanine ammonia- lyase (*PAL*), 4-Coumarate CoA ligase (*4CL*), Cinnamate-4-hydroxylase (*C4H*), Hydroxycinnamoyltransferase (*HCT*), Cinnamate-3-hydroxylyase (*C3H*), O-methlytransferase (*OMT*), Diektide CoA synthase (*DCS*), Curcumin synthase (*CURS*).

4. Novel strategies to augment curcuminoids production

4.1. Molecular markers for identification elite genotypes

Analysis of morphological and phytochemical yield alone is not sufficient for elite genotype identification as the secondary metabolites are subject to variation under different environmental conditions. Though there are many high yielding turmeric cultivars, rigorous identification of the cultivars is essential because of their morphological resemblance (Sahoo *et al*., 2017). Biochemical markers were used to eliminate the duplicate turmeric varieties (Shamina *et al*., 1998). The assessment of turmeric genetic diversity is prerequisite for its breeding programme (Nass, 2001). The different molecular markers techniques which are used for elite genotypes identification in turmeric include random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) (Panda *et al*., 2007; Angel *et al*., 2008), Simple Sequence Repeat (SSR) (Sigrist *et al*., 2010; Joshi *et al*.,



2010; Sahoo *et al*., 2017) and Directed Amplification of Minisatellite DNA (Verma *et al*., 2015). Turmeric is propagatedvegetatively by means of rhizomes so hybridization is mostly unsuccessful. The elite genotypes are typically identified by field trails. Therefore, molecular markers will play a crucial role for germplasm identification and aid in boosting the turmeric quality.

In comparison to the biochemical markers the molecular markers are less influenced by the environment and thus will accurately identify the germplasm (Thimmappaiah *et al*., 2009; Cheng and Huang, 2009). RAPD and ISSR markers mostly widely used molecular markers for genetic diversity analysis (Ebrahimi *et al*., 2009). SSR markers are gaining much more importance than the RAPD and ISSR markers. The problem with RAPD markers is poor reproducibility of banding pattern (Mei *et al*., 2015). Besides, both the RAPD and ISSR markers are dominant and less reliable. The SSR markers offer numerous advantages than the other markers as they are co-dominant with high reproducibility and easy automation (UPOV/INF/17/1 2010 guideline). Moreover, SSRs are mostly two types: genomic SSRs and EST-SSRs. As compared to the genomic SSRs the EST-SSRs are more advantageous as they are derived from the expressed sequenced data and thus can be improve the applicability of genetic markers by expressing the variation in transcribed gene (Scott *et al*., 2000). Several studies have reported the use of SSR markers in cultivar identification and genotyping of different species (Koussao *et al*., 2014; Basheer-Salimia *et al*., 2014). Sahoo *et al*. (2017) have reported the use of EST-SSR marker for the identification and authentication of two high yielding turmeric cultivars (Lakadong and Suvarna). Thus, more studies are needed on this aspect which will enable proper identification of elite turmeric cultivars.

4.2. Evaluation of elite turmeric cultivars by planting at different agroclimatic zones

The demand for curcumin is increasing day-by-day (Li *et al*., 2015). Turmeric cultivars with high percentage of curcumin are attracting the worldwide market. However, variation in curcumin percentage in turmeric when grown at different places restricts the export potential. Though India is the largest producer of turmeric with many yielding turmeric cultivars, the average productivity is not satisfactory (Ayer, 2017). When a high curcumin yielding cultivar is cultivated in places other than its place of origin the curcumin percentage falls remarkably, thus affecting their commercial potential. The curcuminpercent in turmeric cultivars range from 2-7% (Sasikumar, 2005). Anandaraj *et al.* (2014) evaluated the curcumin content of eleven different turmeric cultivars across ten different locations in India for selection of stable genotypes with respect to different environmental parameters. The mean curcumin content of all the cultivars ranged from 4.22% -5.78%. The results from their study indicated that Mega Turmeric, IISR Kedaram and IISR Prathiba were highly stable and could be used in breeding programs for obtaining high dry yield and curcumin content. Significant differences in the morphological characters, essential oil and curcumin of high yielding turmeric cultivars (Surama and Roma) were observed when grown at different agroclimatic zones (Sandeep *et al*., 2016). The variation in the curcumin content was from 1.5-5% (Surama) and 1.4-5% (Roma). Soil nutrients (Nitrogen, Phosphorous and Potassium), Soil pH and altitude were most sensitive factors for curcumin content. Variation in secondary metabolite production with varying soil nutrients has also been reported in other plants (Alam and Naik, 2009; Ramakrishna and Ravishankar, 2011). Kandasamy *et al.* (2012) reported positive effect of potassium on curcumin yield. Gupta *et al*. (2015) have evaluated genetic divergence among 65 turmeric genotypes with respect to thirteen agro-morphological traits. They have grouped the genotypes into seven clusters and reported that the genotypes of cluster VI and cluster VII can be utilized for turmeric breeding. Thus, subjecting high yielding turmeric cultivars to different agroclimatic zones prior to their release will not only help in understanding the best environmental conditions for maximum phytochemical yield but also for managing the soil parameters for enhancing curcumin and essential oil.

4.3. Biotechnology approaches

Curcuminoids have enormous importance worldwide because of their wide range of medicinal properties and the research on curcumin has doubled since the past few years. Like all other secondary metabolites,the curcuminoids are also influenced by environmental factors and are accumulated over long period of time in plants. Besides, they are also subjected to seasonal variation (Fang *et al*., 2017). Furthermore, chemical synthesis of curcumin is costly (Rodrigues *et al*., 2015). To meet the demand of curcumin, there is need for novel strategies whereby the curcumin production can be enhanced. One such strategy that can be employed for enhancing curcuminoid content is heterologous production and metabolic engineering. Both microorganisms and plants can be used as for metabolic engineering. The heterologous production of curcuminoids in microrganisms is mostly preferred because larger amounts can be produced at low time (Lussier *et al*., 2012). The heterologous production in plants is costly and also several complications associated with the genetically modified plants in society. In addition the rules for genetically modified organisms are simpler than crops (Halls and Yu, 2008). Based, upon these facts the microorganisms are more preferred than plants for

the heterologous curcuminoid production. The use of appropriate synthetic enzymes are the key components for successful heterologous production of the desired compounds. The production of curcuminoids can be enhanced by utilization of substitute enzymes from other microorganisms that are well-suited with the heterologous host which permit greater curcuminoid yield. Likewise, more specific enzymes must be identified which will also improve the curcuminoid production with less preferred byproducts.

Katsuyama *et al*. (2008) for the first time reported the production of curcuminoids in *E. coli* by using tyrosine/ phenylalanine as the starter substrate, which is then converted to carboxylic acids by phenylalanine ammonia lyase (*PAL*). The carboxylic acids are converted to CoA esters by 4CL (4-coumarate-CoA ligase) and finally to curcuminoids by curcuminoids synthase (CUS). Acetyl-CoA carboxylase (ACC) was overexpressed for increasing the malonyl-CoA in *E. coli* as malonyl-CoA is also necessary for curcuminoid biosynthesis (Katsuyama *et al*., 2007). Generally the malonyl-CoA in microorganisms is utilized in fatty acid production which is the reason for low availability of malonyl-CoA for recombinant pathways and there are reports whereby ACC overexpression resulted in increase of bisdemethoxycurcumin (BDMC) titers (Xu *et al*., 2011). Till date much of the work on heterologous curcuminoid production is done using *E. coli* as host (Rodrigues *et al*., 2015). Engineering of curcuminoids using *S. cerevisiae* in comparison to *E. coli* offers several advantages as later is an eukaryotic organism and posseses post translation machinery. Besides, the cellular compartment of yeast is also similar to that of plant cell (Jiang *et al*., 2005). In curcuminoid biosynthesis pathway cinnamate-4-hydroxylase (*C4H*) is an important enzyme which converts cinnamic acid tocoumaric acid. In case of *E. coli*expression of *C4H* is challenging but in yeast there is no such problem. Several studies demonstrated the successful cloning and expression of *C4H* in *S. cerevisiae* from different plants (Yan *et al*., 2005; Trantas *et al*., 2009; Shin *et al*., 2012). Fang *et al*. (2017) engineered curcuminoid pathway in *E. coli*and have reported a co-culture technique for rapid production of curcuminoids from glucose. They have used two different strains of *E. coli* for conversion of glucose substrate to curcuminoid and have found out that co-culture led to the greater curcuminoid production than the single *E. coli* strain.

4.4. Artificial neural network (ANN) for predicting the curcumin content

The artificial neural network (ANN) is a mathematical model based upon statistical algorithms which resemble the mammalian neural network. The model consists of three layers of design: input layer in which data is fed, hidden layer in which the data processing occurs and outer layer which generates the result. Because of their high efficiency and accuracy for large data sets, the ANNs have wide range of applications from marketing, industry, finance, medicine and agriculture. ANN was used for predicting the yield of wheat in Argentine Pampas region (Alvarez, 2009). Artificial neural network models were developed for increasing the podophyllotoxin (anticancer compound) yield in *Podophyllyumhexandrum* (Alam and Naik, 2009). Similarly, the yield of sunflower in response to salinity and soil moisture was predicted by ANN model (Dai *et al*., 2011). Akbar *et al*. (2016) developed a prediction model based upon ANN for site specific cultivation of turmeric for maximum curcumin production. Thus, artificial neural network will help to generate high yielding turmeric varieties which will perform consistently across different agroclimatic zones.

5. Future Perspectives

The demand for quality turmeric products world over has triggered the production of this species many folds worldwide. There is an increase in demand for curcumin day by day. Moreover, the demand for rhizome and leaf essential oil is also increasing in the global market. In this article an overview of the impact of environmental factors on secondary metabolite contents in *Curcuma longa* L. is presented. In this context, the identification of elite turmeric varieties and their evaluation by planting at different agroclimatic zones is extremely important. In addition, expression of genes responsible for curcumin production in different environment and soil bears immense potential for future research and development. However, there are several critical factors which need to be addressed in research pertaining to improvement in turmeric production. In addition, more studies on heterologous production and metabolic engineering by employing new hosts are needed which will increase the curcumin production to a greater extent.

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Cell death in *Arabidopsis* mediated by *AtATG6* provides immunity against *Magnaporthe oryzae*

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ABSTRACT

Rice succumbs to *Magnaporthe oryzae*, causing rice blast, while *Arabidopsis*, acting as a nonhost, deploys an active defense mechanism against the pathogen. Despite extensive gene and QTL identification, no cure for rice blast has been found. *Arabidopsis* employs non-host resistance (NHR) with a hypersensitive response (HR) involving localized programmed cell death, crucial for pathogen limitation and resistance. Recognizing the pivotal link between cell death and disease resistance, our study delves into the relationship between AtATG6 and HR-associated cell death, focusing on its role in resisting *Magnaporthe oryzae*. In our experiments, both wild-type Col-0 and ATG6 mutant *Arabidopsis* were exposed to *Magnaporthe oryzae*, with trypan blue staining and electrolyte assays gauging cell viability dynamics. Microscopic representation of the oxidative burst examined the correlation between reactive oxygen species (ROS) generation and cell death. As revealed by the relative expression patterns of defense genes (PR1, WRKY53, and WRKY29), the mutant ATG6 operates by subverting the defense mechanism. Raman spectra analysis uncovered compromised plant immunity, manifesting as variations in carotenoid levels. This study illuminates the intricate interplay of AtATG6, cell death, and disease resistance in defense against *Magnaporthe oryzae* by *Arabidopsis*.

1. Introduction

Plants encounter biotic stress from a diverse array of microorganisms throughout their lifecycle, with only a few posing harm. The evolution of defense mechanisms in plants, shaped by the co-evolution of pathogenic microorganisms and plants, involves the recognition features on the plant's surface (Burdon & Thrall, 2009; Dodds & Rathjen, 2010; Schulze-Lefert & Panstruga, 2011). The vigilance in plant defense limits the compromise of innate defenses by pathogens, resulting in disease induction by only a few (U. Lipka, Fuchs, & Lipka, 2008). Plants exhibit induced resistance that operates at locations distant from the initial infection, providing long-term resistance triggered as an "alert signal" after the first encounter with pathogens. Conversely, successful breaches of inherent defenses during compatible interactions allow pathogen colonization, while active resistance prevents colonization during incompatible interactions (Dangl & Jones, 2001; Gill, Lee, & Mysore, 2015; Thordal-Christensen, 2003).

The decline in staple crop production, particularly rice (*Oryza sativa* L.), is attributed to various biotic and abiotic stresses globally, with fungal diseases causing significant production losses. The rice blast disease, caused by the highly damaging hemibiotrophic fungus *Magnaporthe oryzae*, remains a persistent challenge despite advanced disease management strategies. Developing disease-resistant cultivars through the incorporation of nonhost genes is a viable long-term solution (Devanna *et al.,* 2022; Reddy *et al.,* 2021).

Nonhost resistance (NHR), a broad-spectrum resistance against all genetic variants of a specific disease in a particular

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plant species, relies on self- and non-self-recognition within the plant immune system (Hadwiger, 2015; V. Lipka *et al.,* 2005; Senthil-Kumar & Mysore, 2013). Understanding the mechanisms of NHR and utilizing them for sustainable farming is a priority. NHR involves pre-formed physical and chemical barriers at the pre-penetration stage, with the identification of NHR genes and their molecular features being crucial for developing disease-resistant crop varieties (da Cunha, McFall & Mackey, 2006).

The intricate plant immune system encompasses various responses, including pathogen-induced cuticular wax synthesis, reactive oxygen species (ROS) production, and hypersensitive cell death. Plant recognition of pathogen-associated molecular patterns (PAMPs) initiates PAMP-triggered immunity (PTI), the first line of defense. Effector-triggered susceptibility (ETS) occurs when pathogens suppress PTI, leading to susceptibility in hosts. The zig-zag model illustrates the interplay between effector-triggered immunity (ETI) and PTI, involving cellular processes such as MAP kinase signaling, ROS production, and hypersensitive response (HR) (Dodds & Rathjen, 2010; Jones & Dangl, 2006; Zurbriggen, Carrillo, & Hajirezaei, 2010).

The genetically tractable Arabidopsis system provides insights into innate immunity and programmed cell death (PCD) pathways, including autophagy. Recent data from autophagy-deficient Arabidopsis suggest a significant role for autophagy in controlling plant immune responses, although its precise role remains unclear (Talbot & Kershaw, 2009; Yoshimoto *et al.,* 2009). Autophagy-related (ATG) genes, including ATG6, are involved in this catabolic process, impacting plant stress responses and pathogen-induced cell death (Hayward & Dinesh-Kumar, 2011; Hofius *et al.,* 2009).

ATG6, part of a complex with class III phosphatidylinositol T-kinase (PI3K)/Vps34, plays a role in vacuolar protein sorting (VPS) and autophagy in yeast. Plant ATG6 deficiency reduces autolysosome production, leading to increased susceptibility to stressors. ATG6's involvement in autophagy suggests its potential role in agricultural improvements and immunity-associated plant cell death (Furuya *et al.,* 2005; Patel & Dinesh-Kumar, 2008).

While the molecular mechanisms of autophagy and ATG6-associated pathways have received limited attention in crop plants, understanding these processes in nonhost model organisms may hold the key to enhancing disease resistance and agricultural productivity (Edinger & Thompson, 2004; Greenberg & Yao, 2004; Levine & Klionsky, 2004). In the present study, we delved into the molecular mechanisms through which AtATG6 orchestrates autophagy and oversees hypersensitive response-programmed cell death (HR-PCD) in the innate immune response within the nonhost, specifically *Arabidopsis*. Our findings reveal that ATG6 plays a crucial role in conferring immunity, linked with plant cell death, and holds the potential to be a key factor in advancing agricultural practices.

2. Materials and Methods

2.1 Plant growth and maintenance:

The *Arabidopsis thaliana* wild ecotype Col-0 (N1093) and the ATG6 mutant (N678948; homozygous for its T-DNA insertion) were sourced from the Nottingham *Arabidopsis* Stock Centre (NASC) and cultivated in a plant growth chamber. Ten-day-old seedlings were grown on flats containing a mixture of agropeat and vermiculite soil (3:1) under controlled conditions: light maintained at ~100 µE/m^2/s, temperature at 22°C, humidity at 65%, with a 14-hour light: 10-hour dark cycle. The growth medium was supplemented with ½ strength Hoagland growth media. Leaves from 21-day-old seedlings were utilized for the infection assay. Seeds from the respective ecotype and mutant were harvested and stored at 4°C for future use.

2.2 Pathogen culture conditions:

M. oryzae spores were collected from the National Rice Research Institute (ICAR-NRRI) and grown on freshly prepared oatmeal agar (OMA) plates at 28^0 C until sporulation (~7-9 days) for use during infection assay. The spore blocks from the old stock were transferred to the freshly prepared OMA medium in every 7-10 days. Antibiotic streptomycin (100µg/mL) was used to avoid any bacterial contaminations.

2.3 Infection assay:

The detached leaf assay for infection was performed by taking three leaves of 21 days old seedlings of soil grown *Arabidopsis*. Leaves of wild type and mutant ecotype of *Arabidopsis* were inoculated on right side of leaves beneath mid rib with 10 µL of conidia (approximately 10^5 spore/mL) extract of *M. oryzae* in 0.01% tween 20 solution. As a control, 10 µL drop of 0.01% tween 20 solution was put on the leaves. Three upper rosette leaves from each seedling were detached and kept on the moist filter paper in petriplates to maintain 100% humidity that is suitable for infection by the rice blast fungus. It was further covered by the Petri dish and kept under dark for a day and was exposed to light thereafter until disease progression was experimented. The phenotypes were observed at 1 DPI and 3 DPI. Three experimental set-ups were used for each ecotype.

2.4 Trypan Blue Staining:

The inoculated leaves were harvested for staining with

trypan blue to observe cell viability at desired time points. The inoculated leaves were kept dipped in alcoholic lactophenol for around 24 hours in the cups of 24 well microtiter plate until chlorosis. Leaves in the microtiter plate were stained with trypan blue (250 µg/mL) made in lactophenol (phenol: glycerol: lactic acid: water 1:1:1:1, v/v) for 15 minutes. It was further destained with lactophenol for ~1 hour; mounted in 50% glycerol and examined under bright field microscope (Vogel & Somerville, 2000).

2.5 Electrolyte leakage assay:

Two leaf discs of each ecotype (both control and treated) were cut and immediately put into well plates containing 2mL of sterile distilled water. Covered the plate with lid and incubated for 30minutes in a growth chamber. Then the water was replaced in each well with 2mL fresh sterile water. Again, it was incubated for different time intervals in the growth chamber. Calibrated electrolytic conductivity meter was used to check the conductivity of the solution. Then after, 100uL water sample from each well were put on the conductivity meter (LAQUAtwin-EC-33, HORIBA Scientific) and measured for the electrolyte conductivity at the determined time points (Jamra *et al.,* 2021).

2.6 DAB staining:

Diaminobenzidine (DAB) staining was performed to stain the reactive oxygen species (ROS) in early hour of infection (Daudi & O'Brien, 2012). Firstly, the treated leaves were soaked in DAB staining solution (1mg/mL) for 12hr. Then it was replaced by distilled water and kept for 12hr. Further, the leaves were dipped in solution of ethanol: acetic acid (96: 4) for chlorosis. After destaining, leaves were observed under brightfield microscope and images were recorded in an inverted light microscope (Magnus, Magcam-DC5 and OLYMPUS) for screening.

2.7 RNA (Ribonucleic acid) extraction and RT-qPCR analysis

The extraction of RNA from the control and treated leaves of *Arabidopsis* accessions were done using the TRIzol reagent following manufacturer's instructions. RNA concentration and purity was measured by the help of Nanodrop and 1.2% agarose gel electrophoresis, respectively. DNase I treatment was employed to remove any genomic DNA (Deoxyribonucleic acid) which was further confirmed by performing -RT-qPCR reaction (Actin primer set was used, gDNA amplification size is 220 bp and cDNA amplification size is 134 bp). Two µg of RNA samples were further processed to synthesize cDNA using cDNA synthesis kit. The level of expression of *PR1, WRKY53* and *WRKY29* marker genes were recorded and normalized to the expression level of *AtACTIN2* as internal control (Table 1).

2.8 Raman Spectroscopy

Fresh inoculated leaflets were collected at 3dpi and positioned on the stage mount onto a glass slide and used to take Raman spectral reading. Treated leaves were punched from the selected infected areas. The leaf samples were placed on the glass slide. The Raman measurement conditions were 800–1800 cm^{-1} of spectral range, 10 s of acquisition time, 20mW laser power, 532 nm visible light band, 1200gr/mm grating, 100µm slit, 300µm hole and 20x magnification objective (micro spot with 10µm ø). The calibration was performed daily by recording the Raman signal of a silicon wafer. In total, 3 biological replicates of spectral data sets were obtained from each control and infected plants. Raman spectra shown in this work correspond to the raw baseline corrected results along with smoothing of line graphs using Origin Pro 8.5 software. (Butler *et al.,* 2016; Vallejo-Pérez *et al.,* 2021).

2.9 Statistical analysis

Origin Pro 8.5 was used to calculate the Raman shifts obtained as raw data from the measurement of the spectra for baseline correction and deducing the graph. Graph Pad Prism 8.0.1 was used to prepare the fold changes in the expression of the differentially expressed genes under stress.

Table 1. List of primers used in qRT-PCR

Gene	Forward Primer	Reverse Primer
AtACT2	TCGGTGGTTCCATTCTTGCT	GCTTTTTAAGCCTTTGATCTTGAGAG
PR1	AAAACTTAGCCTGGGGTAGCGG	CCACCATTGTTACACCTCACTTTG
WRKY53	ACACCACCATTAGCCTCGCC	ACGCGGGGAAAGTTGTGTCA
WRKY29	CGGAG*ATG*GAGACAAGTGGCTT	TGTGAGGATCGTTTGTGTGGAGAA

3. Results

3.1 Arabidopsis *mutant* ATG6 *exhibits breach in immunity against* M. oryzae

The autophagy protein 6 encoded by *AT3G61710* functions for autophagosome assembly, mitophagy and protein targeting to vacuole that is meant for inducing programmed cell death in *Arabidopsis* (Feng, De Rycke, Dagdas, & Nowack, 2022; Lai, Wang, Zheng, Fan, & Chen, 2011; Lee et al., 2018). During pathogen attack, the plants sense the invader and counteract with several defence mechanisms according to the severity of infection. This defence response includes cell death restricting the pathogen spread in the host tissues. With the purpose of finding out the role of *AtATG6* in disease resistance against rice blast we used the homozygous T-DNA insertion line to check its possible involvement relating to disease resistance.

To study the interaction and pathogenicity of *M. oryzae* in wild type *Arabidopsis* Col-0 and mutant *ATG6,* trypan blue staining was performed. Unlike in Col-0, the pathogenicity of *M. oryzae* in *ATG6* with a susceptible response as early as 1 dpi was observed as evidenced from appressoria and hyphae formation. Trypan blue stain confirmed increased number of appressoria, heavy mycelia growth of the pathogen along with higher cell death due to hyphal penetration in *ATG6* 3 dpi leaves as compared to Col-0 (Fig. 1). This showed attempted but failed pathogen penetration inside the cell. This depicts the rise in hypersensitive cell death in *ATG6* at the entry site the pathogen during infection. Hence, this result confirms the contribution of *AtATG6* in offering nonhost resistance to the plant.

3.2 Ion conductivity enhanced in ATG6 *due to impaired immunity*

During pathogen attack the cell membrane integrity is somehow disrupted or completely lost due to cell death. This triggers higher chance of ion leakage from the cell. The computation of cell death from detached leaves of Col-0 and *ATG6* challenged with *M. oryzae* at 1dpi, 2dpi and 3 dpi using an ion leakage test was consequently of interest. Upon normalizing the measured ion leakage values (µ&!$^{-1}$) to the corresponding water control, the treated leaves of *ATG6* demonstrated increased cell death in comparison to Col-0 (Fig. 2). The infection by *M. oryzae* caused *ATG6* to have weakened immunity at increasing time points. The data are consistent with the trypan blue staining assay and can be correlated.

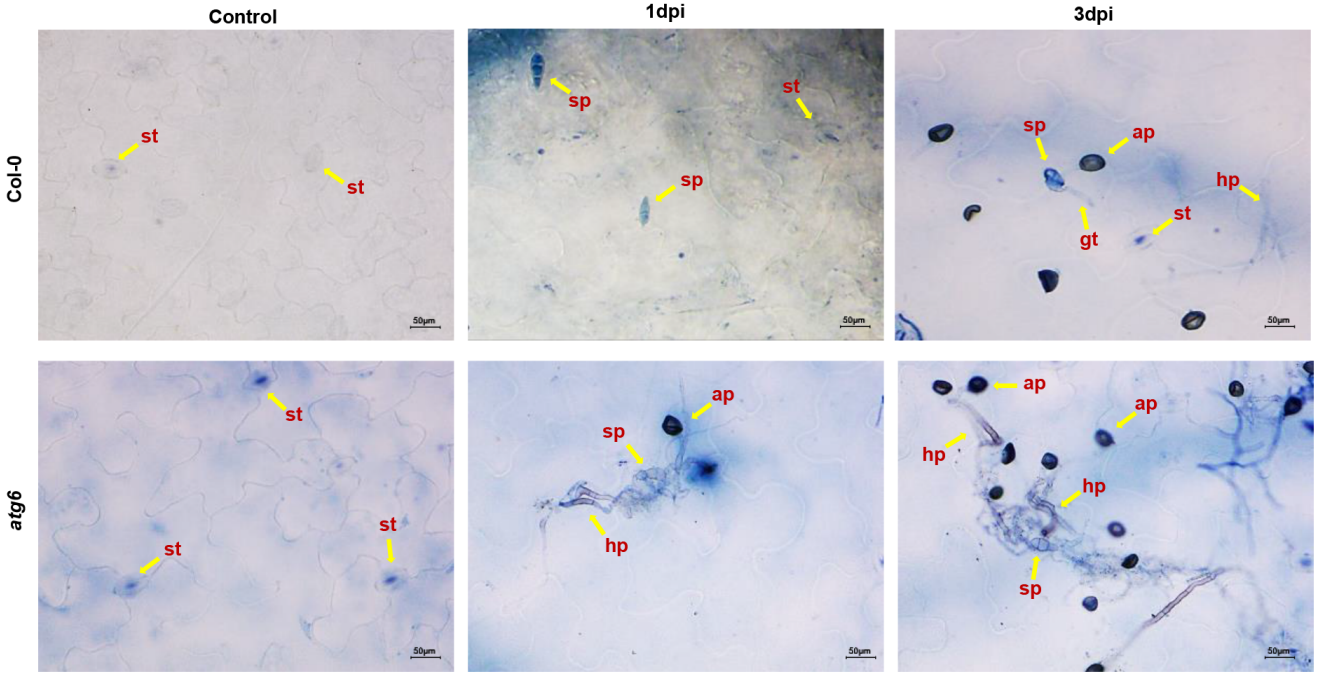

Figure 1: Trypan staining of infected leaves of Arabidopsis. Differential cell death in Col-0 (WT) and *atg6* (mutant) using trypan blue staining at 1 and 3 dpi. st, stomata; sp, spore/conidia; ap, appressoria; gt, germ tube. Scale bar =50µm

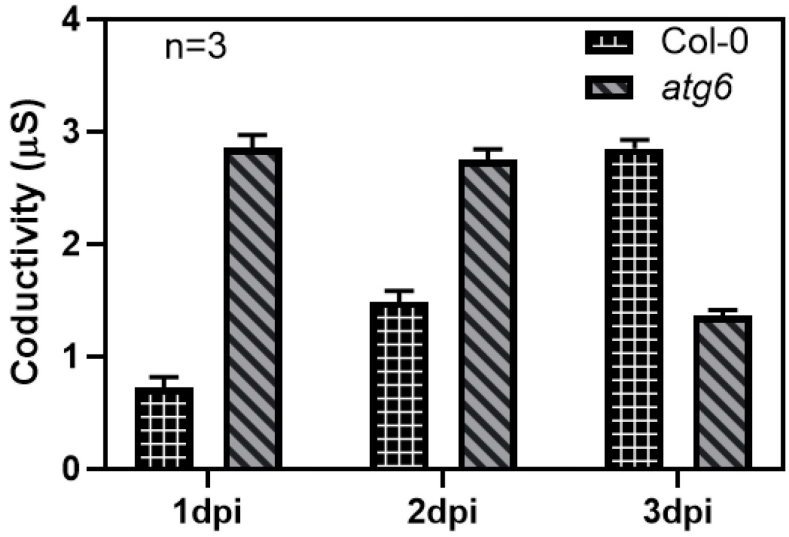

Figure 2: Electrolyte conductivity of infected leaves of Arabidopsis. Differential electrolyte leakage in Col-0 (WT) and *atg6* (mutant) measured at 1, 2 and 3 dpi. Equal area was used to measure the leakage. Three independent biological replication was used to calculate the SD.

3.3 ATG6 undergoes oxidative burst upon infection with M. oryzae

At the site of plant-pathogen contact, the production of ROS by plants serves as an early defence mechanism against biotic stress (Torres, 2010). As a result, DAB staining was performed to track the build-up of H_2O_2 as a yellowish-brown stain. Compared to Col-0, *ATG6* exhibited a noticeably higher level of H_2O_2 generation, as we discovered. Thus, an increase in the hypersensitive response brought upon pathogen invasion is associated with increased H2O2 generation (Fig. 3).

3.4 Differential expression of defence related genes

Activation of various molecular and physiological changes in plants are responses after sensing of challenges from intruding pathogen. Following pathogen infection, host synthesises several signalling cascades at various levels of defence. These signalling molecules includes various

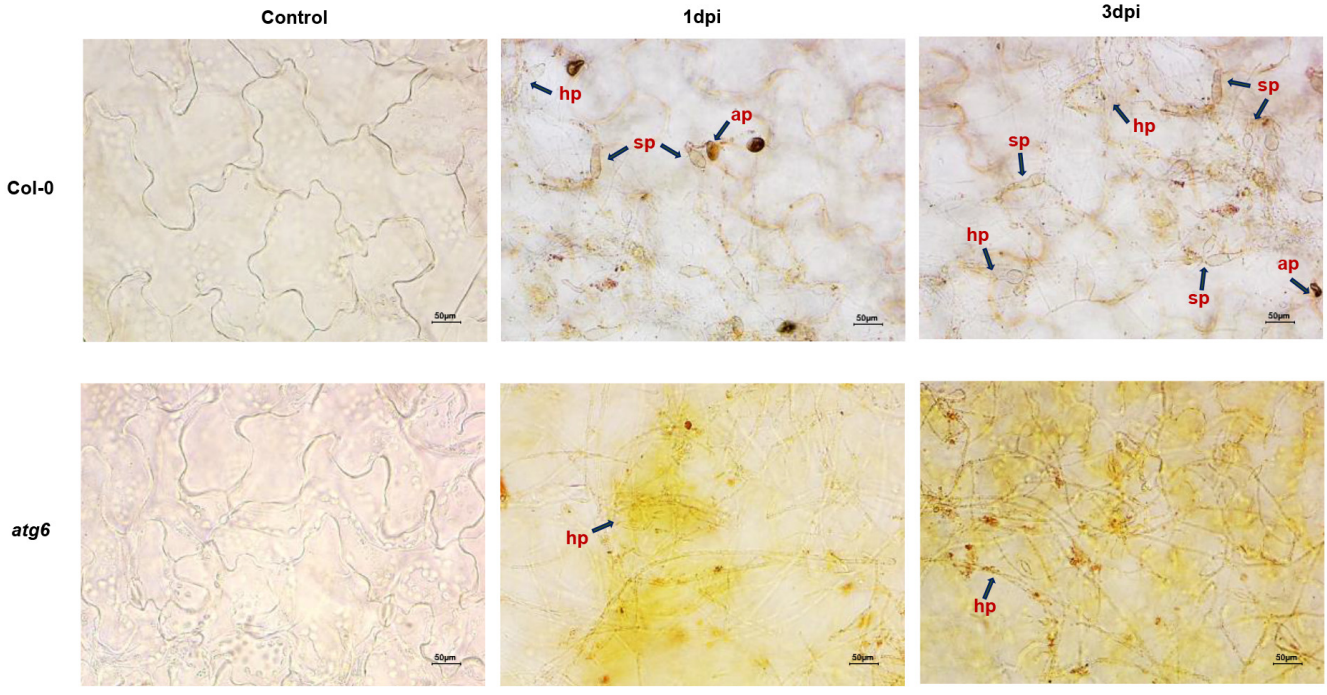

Figure 3: DAB staining of infected leaves of Arabidopsis. Differential ROS generation in Col-0 (WT) and *atg6* (mutant) using DAB staining at 1 and 3 dpi. St, stomata; sp, spore/conidia; ap, appressoria; hp, hyphae. Scale bar =50µm

hormones like salicylic acid (SA), jasmonic acid (JA), and ethylene as initial mode of defence. At molecular level, many defence related genes are activated in host plants further triggering the plant immune responses such as pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI) (Gill *et al.,* 2015; He *et al.,* 2007; Rezaei, Mahdian, Babaeizad, Hashemi- Petroudi, & Alavi, 2019). Among the defence marker genes, *PR1* and *WRKY53* are responsible for early defence that is PTI, whereas *WRKY29* is involved in hypersensitive responses in later stages of infection (Hönig, Roeber, Schmülling, & Cortleven, 2023; Jiao *et al.,* 2022; Yi, Shirasu, Moon, Lee & Kwon, 2014). The relative expression level of *PR1* and *WRKY53* in *ATG6* in contrast to Col-0 increased after pathogen attack while expression of *WRKY29* is like or slightly higher than in Col-0 which depicts the elevated PTI responses in *ATG6* unlike in Col-0.

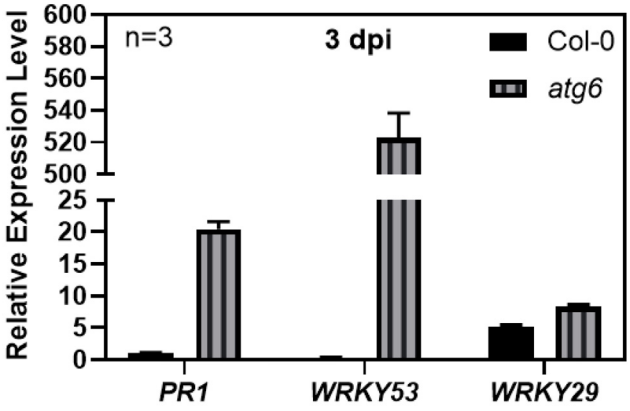

Figure 4: Differential expression of defence markers using qRT-PCR. Representation of the differential expression of genes *PR1*, *WRKY53* and *WRKY29* in Col-0 (WT) and *atg6* (mutant). Actin was used as internal control. Three independent biological replication was used to calculate the SD.

3.5 Spectral differences between Col-0 (WT) and ATG6 *indicate the differential expression of biomolecules involved in ROS chelation and other responses*

The Raman spectra obtained from *Arabidopsis* ecotypes of water control and infected leaves exhibited peaks associated to cellular components, and most prominent vibrational bands were associated to carbohydrates, carotenoids, chlorophyll, and phenolic compounds (Butler *et al.,* 2016; Chen, Zeng, Larkum, & Cai, 2004; Qin, Chao, & Kim, 2012). The plant-pathogen interaction is a complex biological system which can manipulate the plant metabolism and evade defence responses. Thus, the biochemical alterations were induced during the *M. oryzae* invasion, and these shifts were detectable in the Raman spectra (Mandrile *et al.,* 2019; Picaud, Le Moigne, Gomez de Gracia, & Desbois, 2001). The observed shift in peaks suggests degradation of carotenoids in the mutant *ATG6* due to breach in immunity as compared to Col- 0 (Figure-5).

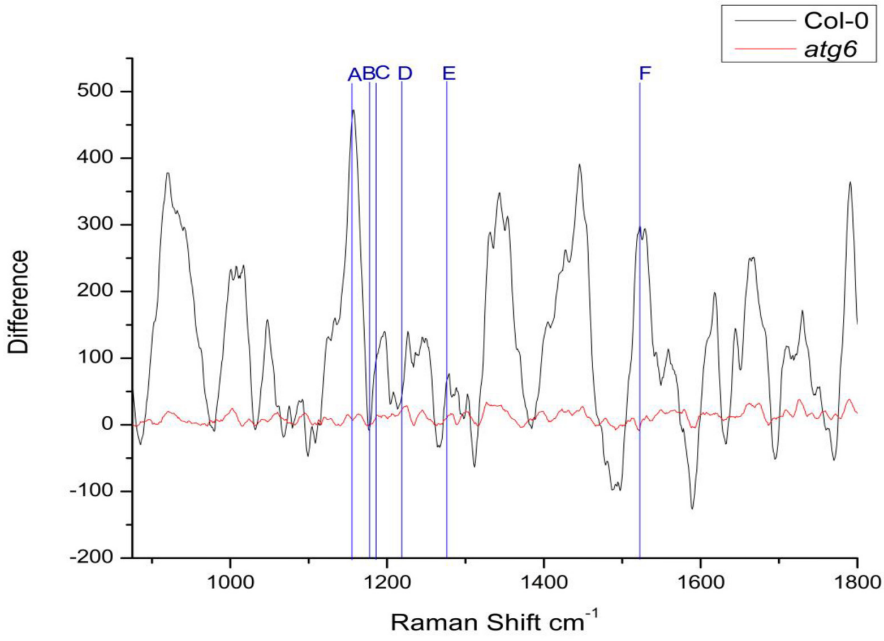

Figure 5. Raman spectra of the Arabidopsis infected with *M. oryzae* conidia. The difference in Raman spectra was calculated by deducting the value of control from the Infected. The raw data obtained from Raman Spectra was corrected with its base line. Vertical lines represent the specific compounds of carotenoids. A- 1155, B- 1180, C- 1185, D- 1218, E- 1276, G- 1521 (Vallejo-Pérez, M. R., et al. (2021); Zeng, J., et al. (2021))

5. Discussion

In the present investigation, we have observed that a mutation in AtATG6 leads to a breach in the plant immune system, resulting in hypersensitive cell death at the sites of pathogen entry (Patel & Dinesh-Kumar, 2008; Xu *et al.,* 2017). This increase in cell death at the infection zones subsequently induces electrolyte leakage, as ATG6 compromises cell membrane integrity (Kacprzyk, Dauphinee, Gallois, Gunawardena, & McCabe, 2016). Microscopic examinations reveal heightened ROS generation in the challenged leaves of ATG6, suggesting the crucial role of ROS as a signaling molecule in defense reactions (Gechev, Van Breusegem, Stone, Denev, & Laloi, 2006; Suman *et al.,* 2021). Consequently, compromised immunity in ATG6 contributes to elevated expression levels of defense genes such as PR1, WRKY53, and WRKY29 (Hönig *et al.,* 2023; Jiao *et al.,* 2022; Yi *et al.,* 2014). Changes in the Raman spectra indicate significant degradation of plant compounds like carotenoids and chlorophylls in pathogen-challenged plants compared to their controls (Zeng *et al.,* 2021).

Collectively, our results lead to the conclusion that cell death mediated by AtATG6 plays a crucial role in disease resistance against *M. oryzae*, contributing to the safeguarding of plant immunity. It is imperative to unravel the core mechanisms of AtATG6 and associated defense genes in conferring resistance to the host plant, elucidating the interconnected pathways involved.

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Traditional rice cultivars grown in Hirakud dam reservoir: Source of submergence tolerance gene under waterlogged environments

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ABSTRACT

In view of the recent climate change scenario which is responsible for creation of multitude of abiotic stresses, development of stress tolerant/resistant crop genotypes is of immense significance for sustainable crop production under stress-prone ecosystems. Submergence or waterlogging is such a harmful abiotic stress factor which can have an adverse influence on rice crop throughout the growing season leading to severe yield losses to the farmers. Hirakud dam reservoir is a man-made water body where water accumulates during the rainy season leading to submergence which is unsuitable for cultivation of any crop and making large land masses unproductive. However, the local farmers live in the embankment areas of the dam reservoir cultivate a large number of submergence tolerant traditional rice varieties exhibiting a lot of variations in their stress resistance mechanisms. Though a huge diversity of traditional rice varieties are cultivated in the dam reservoir, no systematic study on identification of submergence tolerant genes have been carried out. Thus, a proper scientific investigation on this rice diversity will definitely be helpful in providing a source of rich gene pool to develop submergence-tolerant rice genotypes for exploitation of large water bodies for sustainable rice cultivation and making them agro-ecologically productive.

1. Introduction

Rice (*Oryzasativa* L.) is the staplefood for more than half of the World population (Zhao *et al.,* 2017; Jang *et al*., 2023) sustaining life in many developing and underdeveloped Nations across the Globe. In Asian countries, rice cultivation provides employment to majority of the people for maintenance of their livelihood. Most of the rice eaters live in low-income countries of the Asia where more than 76% of caloric intake was obtained from rice only (Panigrahi *et al.,* 2021). Before early 1960s, only traditional tall rice varieties were cultivated in almost all countries of the World where yield was very low and often unable to meet the food demands of human beingsleading to hunger and starvation. In order to increase the yield potential of the plant, people used nitrogenous fertilizers. However, the result was not promising because the plants became excessively tall in response to nitrogenous fertilizers and lodged on the ground causing poor grain filling and reduction in grain yield. Further, the poor yield in the traditional rice varieties was attributable toutilisation of most of the photosynthates for the growth and development of vegetative structures like stem, leaves and roots and very little fraction of photo-assimilates were partitioned into the grains. In the subsequent time of investigation for increasing the grain yield, rice breeders especially at the International Rice Research Institute, Philippines introduced some dwarfing genes in the tall *indica* varieties and became successful in developing the first ever semi-dwarf high yielding miracle rice variety 'IR-8' during early 1960s. This was possible because of the translocation of photosynthates to the grains at the cost of vegetative structures as the plant height was significantly reduced leading to improved harvest index. Subsequently, several IR-8 parented rice varieties were developed through traditional breeding and distributed among the farmers of developing and underdeveloped countries of the World for cultivation in their own farms leading to a quantum jump increase in rice production.

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Though grain production during 1960s was significantly increased, rice production has almost stagnated during the last 2-3 decades and farm yield seldom gone beyond 10 t ha^{-1} (Panigrahi *et al.,* 2021) and thus failed to provide food security in many developing countries as there was a significant increase in population growth. Currently speculation is ripe for a severe shortage of food grains in near future which may bring starvation due to lack of another quantum jump in rice yield potential (Panigrahi *et al*., 2021). Hence, in order to feed all the mouths of the burgeoning population, attempts are to be made to find out new yield thresholds and in this regard, huge biodiversity of traditional submergence-tolerant rice varieties grown in the embankment areas of Hirakud dam reservoir may provide a rich source of gene pool for their introgression into high yielding rice genotypes for exploitation of water resources under waterlogged environments for making those are a sagro-ecologically productive and also for enhancement of farmer's income.

2. Hirakud dam reservoir as the habitat of submergence tolerant rice cultivars

Hirakud dam is the longest earthen dam in the World builtover the river Mahanadi at Hirakud and is located in Sambalpur district of Odisha, India having latitude 21.32N and longitude 83.52E. The dam reservoir is a multi-purpose artificial water body constructed just after India's independence which covers a large land area both in Odisha and Chhattisgarh. Huge water accumulation over the soil surface in the embankment areas occurs during the rainy season which makes the habitat unsuitable for cultivation of any crop including high yielding rice. However, the farmers living in the embankment areas of the dam reservoir cultivate some traditional rice cultivars under such waterlogged conditions since long time. (Figure 1) These varieties are tall in stature and are highly tolerant to submergence and stagnant flooding. The dam reservoir area remains almost dry during the summer season and following the first rain fall, farmers sow the seeds just after ploughing the land before the onset of monsoon. Subsequently seed germinates after the first rain and vegetative growth of the seedlings is quite good because the reservoir soil is highly fertile due to deposition of silt, minerals and other organic matters carried through small rivers and streams during the previous rainy season. At the onset of rainy season, heavy rainfall causes excessive accumulation of water in the dam reservoir and some of the rice varieties rapidly elongate their stem internodes and leaf sheaths to keep some of the leaves above the water level to carry out normal photosynthesis especially during the vegetative growth phase, so that photo-assimilates are synthesized in the leaves which help in the survival of the plant. The photosynthates act as the source of energy to combat against the submergence-induced stress for plant survival. This mechanism of resistance to flooding stress is known as 'escape strategy' (Bashar *et al*., 2019). Submergence-induced internodal elongation is also called 'floating ability' and is mostly evoked by the plant hormone ethylene (Kende *et al*., 1998). Apart from stress escape strategy, some rice cultivars survive completely being submerged for longer time even for over amonth. These varieties continue to grow luxuriantly well after the water level recedes and this strategy of flooding resistance is called 'quiescence strategy' (Oe *et al*., 2022). In such situations, plants experience hypoxia or anoxia stress because of restriction in diffusion of oxygen into the lower parts of the plantthat are completely under water (Mondal *et al*., 2020; Mital *et al*., 2022). Increase in plant height and water depth in thenatural habitats of the Hirakud dam reservoir for the four rice genotypes studied during the wet season of 2017 is presented in Fig.1

3. Waterlogged environments in dam reservoir: Stressful habitats for rice

Water logging or flooding is the third most vital abiotic constraints for crop production after heat and drought (Oladosu *et al*., 2020) and rice is one among the most flood-threatened crop. About 30% of the marginal farmers and poorpeoplelive in the flood-prone areas of South Asian countries including India, Nepal and Bangladesh. In Indian situation, 5.2 million hectares of land areas are affected by occasional flood, out of the total 16.1 million hectares of rice growing areas and about 700 million people live in flood-prone rice cultivating areas of South Asia (Oladosu *et al*., 2020; Jang *et al*., 2023). More than 35% rice cultivable areas especially in African and Asian countries are prone to flooding stress where food insecurity is a major issue (Bailey-Serres *et al*., 2012). In fact, flooding is a major constraint that threatens the human livelihood as it has tremendous influence on the vulnerability and poverty in the marginalised rural population of Africa and Asia.

The stressful habitats created through water logging or submergence is often developmental stage specific. Submergence during seed germination called anaerobic germination provides an anoxic or hypoxic environment causing low or poor germination and poor crop establishment or even death of the entire seedlings due to reduction in ATP production for maintenance of metabolic activities. On the other hand, if it is a fully grown submerged plant, it may lodge on the water surface after the flood subsides and may die eventually due to depletion of reserve carbohydrates and cellular energy (Oladosu *et al*., 2020) leading to hindrance of growth and development. Rainwater flooding

Figure 1 : Rice cultivation in Hirakud dam reservoir.

is generally clear water which causes less crop damage than muddy and silted water as the latter becomes turbid and largely inhibit the entry of light into the submerged leaves leading to poor photosynthesis and has a considerable impact on the physiological status of the plant. Moreover, continuous water logging for longer duration causes chlorophyll and protein degradation, decrease of Rubisco activity and damage to photosynthetic apparatus leading to drastic impairment of photosynthesis (Panda *et al.,* 2008). Both oxygen deprivation and low solar intensity under prolonged water-logged situation are responsible for poor plant survival due to failure in new leaf production and severe damage of the older leaves. In addition to this, submerged plants produce a varieties of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical, superoxide anion and superoxide radical which have severe damage potential to the cellular organisation leading to death of the entire plant. However, several rice plants grown in the Hirakud dam reservoir have tremendous potentiality for detoxification of such ROS in their natural habitats (data not shown). Under complete submergence, rice plants tend to elongate the stem internodes quickly in response to high ethylene production and oxygen deprivation. However, re-oxygenation after a period of oxygen deprivation, ethanol produced from glycolysis-derived pyruvic acid through anaerobic respiration (alcohol fermentation) is being trapped in the plant tissue and subsequently converted to acetaldehyde which is responsible for severe post-anoxic cell damage (Voesenek *et al*., 2013; Oladosu *et al*., 2020). Generally, one glucose molecule can produce approximately 2 ATPs under anaerobic respiration whereas 38 ATPs are produced from the single glucose molecule during aerobic respiration and therefore plants under complete submergence face an acute problem of energy shortage because of very quicker consumption of the respiratory substrates like glucose and other simple carbohydrates (Lee *et al.,* 2019) leading to starvation.

4. Varietal diversity of submergence-tolerant rice cultivars in the dam reservoir

There was a huge diversity of traditional rice cultivars grown in the embankment region of Hirakud dam reservoir. However, the diversity has been lost at present due to discontinuation of cultivation of these varieties by the farmers as their grain yield is very low and also shortage of water occurs especially during the harvest period. Many cultivars grow upright as water level is high, but lodge by bending their stem internodes, if water level is decreased leading to death of the whole plant. Construction of many barrages on the upstream of river Mahanadi is also one of the principal reasons for problem in water accumulation in the rice growing regions especially during post-rainy

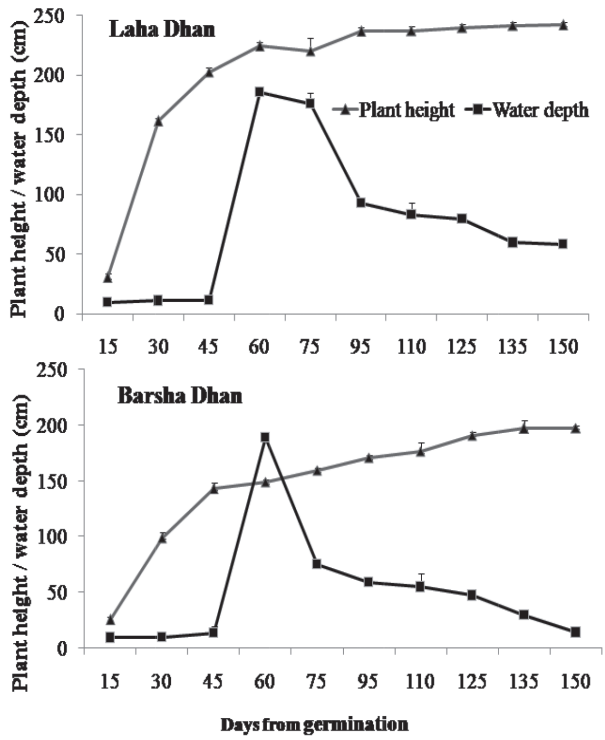

Figure 2: Plant height (triangle) and water level (square) in the natural habitats of Hirakud dam reservoir for the submergence escape rice cultivar *LahaDhan* (upper panel) and submergence quiescence rice cultivar *BarshaDhan*grown during the wet season of 2017. Vertical bars represent ± SD values (n=3).

seasons at the time of crop harvest. As a result of water shortage, the upper part of the stem becomes weaker and lodge on water surface leading to poor grain filling for which there is significant yield loss. In spite of this, many farmers in the villages like Chikili, Gudam, Sardha, Kankel, Remta, Lora, Bhadrapali, Ganthipali and many others located in the embankment regions of dam reservoir both in Odisha and Chhattisgarh still cultivate some rice varieties popularly known as *Budi dhan*. Out of these,varieties like *Laha dhan*, *Laha dumber*, *Lahagayanti*, etc. have been collected which follows escape strategy for submergence tolerance whereas *Barsadhan*, *Budi dhan*, *Hatipanjer*, *Khudiadhan*, *Radhajugal* etc. exhibit 'Quiescence strategy' of submergence tolerance.

5. Ethylene as the key controller of submergence tolerance in rice

Although the rice crop needs huge quantities of water for successful growth and development, exposure to continuous waterlogged conditions for long time is highly detrimental to the plant as the roots have to face the submergence-induced anoxia. However, several traditional rice cultivars are being cultivated in the upstream of the reservoirwhere the plants are subjected to continuous submergence for longer period. Many cultivars utilise their food reserves like carbohydrates for the purpose of stem elongation by sacrificing few leaves at the basal region of the plant and keep some leaves above the water surface to carry out normal photosynthesis. (Figure-2) Some of the rice varieties can elongate their stem up to 20 cm per day. Plant hormone ethylene plays the key role resulting into spindly plants which can easily lodged on water surface when water level recedes leading to poor survival and producing little or no grain yield. However, these genotypes are important in lowland submerged areas where flood water does not recede for longer time and hence plant produces a good grain yield. Additionally, several genes related to escaping the submergence stress are expressed in these plants and plant hormones like ethylene, GA and ABA play pivotal role in their expression. Under deep water situation, ethylene accumulation promotes the expression of *SNORKEL* (*SK*) genes like *SK1* and *SK2* (Hattori *et al.,* 2009) through the activation of AP2/ ERF transcription factors which are responsible for stem elongation due to activation of GA signalling pathways and down-regulation of Brassinosteroid (*BR*) biosynthesis genes (Bashar *et al*., 2019) (Pathway I). However, *SK* genes are not expressed in all rice varieties and the expression is restricted to lowland deep water accessions only for stem elongation (Bashar *et al*., 2019). In the second pathway, ethylene induced ERF transcription factors also enhances the expression of *Sub1* gene responsible for over accumulation of *Slender Rice-1 (SLR1)* and *SLR1-Like-1* (GA signalling repressors) genes leading to reduction in GA accumulation and inhibition in leaf sheath and internode elongation and thus adopting 'Quiescence strategy'. (Figure-3) So, ethylene is the key factor responsible for induction of GA repressor genes and offset of GA signallingpathway where *Sub1* mediates the entire pathway resulting into inhibition of stem internode elongation in the Quiescence strategy (Fukao *et al*., 2006). In addition to the ethylene induced AP2/ ERF mediated expression of *SK* genes for controlling submergence escape, ethylene can also induce the expression of *OsEIL1a* (*Ethylene Insensitive 3-like 1a*) under submergence stress which binds to the promoter of *SK1/SK2* genesleading to accumulation of *SK1/SK2* transcript and is responsible for down-regulation of BRs and activation of GA (mainly GA1) signalling for rapid intermodal elongation for plant survival under escape strategy (Fukao *et al.,* 2012; Seo *et al*., 2006) (Pathway III). Accumulation of GA1 activates the expression of cyclin genes which causes rapid cell division for elongation of the stem internode in lotus (*Nelumbonucifera*) for adoption of escape strategy (Wang *et al.,* 2018) and such mechanisms may also exist in rice. Apart from *Snorkel*-dependent internode elongation, *Snorkel*-independent stem elongation for escape strategy during submergence is basically mediated

Sampling of Deep-water rice from Hirakud dam reservoir

by ethylene-induced *OsEIL1a, a* protein which binds to the promoter of another gene called *SD1*(*Semi-dwarf1*) that functions in independent of *Snorkel*and promotes the synthesis of bioactive GA especially GA4 leading to more rapid stem elongation for submergence escape (Kuroha *et al*., 2018). Generally, the SD1 protein is responsible for the biosynthesis of bioactive GA like GA4 in addition to GA1 after submergence and GA4 is more efficient in stem elongation that of GA1. Hence *Snorkel*-independent stem elongation mediated by SD1 is relatively faster in comparison to *Snorkel*-dependent pathway (Bashar *et al.,* 2019) for escaping the stress effects of submergence.

A number of research work revealed that, submergence tolerance of many rice genotypes are also mediated by the expression of a major QTL called '*Submergence1*' (*Sub1*) present in the 9th chromosome of rice which encodes an *ethylene response factor* (*ERF*) and is responsible for restriction of stem elongation under submergence and thus adopt quiescence strategy. This *Sub1* QTL contains three genes such as *Sub1A, Sub1B* and *Sub1C* and this QTL has been isolated and characterisedinitially from a traditional rice land race *FR13A* grown in coastal Odisha in India (Xu *et al.*, 2006). Out of these three genes, *Sub1A* is mainly associated with submergence tolerance in rice because *Sub1B* and *Sub1C* are also expressed in the rice varieties which are not tolerant to submergence stress. Under complete submergence, prominent up-regulation of ethylene response factor (ERF) transcription factors genes like *ERF66* and *ERF67* occurs in presence of SUB1A-1 as suggested by Lin *et al.* (2019). In fact,SUB1A-1 is classified as Group-VII ethylene responsive factor (ERFVII) family and confers submergence tolerance through 'quiescence strategy' mediated by repression of ethylene and GA induced stem elongation (Lin *et al*., 2023). So ethylene is indirectly responsible for repression of GA signallingand reduction of GA-mediated gene expression under submergence stress in a SUB1A-1dependent manner. It is suggested that two ERFVII genes like *ERF66* and *ERF67* are transcriptionally activated by SUB1A-1 and a regulatory complex is formed consisting of SUB1A-1 and ERF66/ERF67 for the activation of some downstream genes in rice to obtain submergence tolerance (Lin *et al*., 2019; Lin *et al.,* 2023). It is also proposed that the 186th serine of SUB1A-1 protein is phosphorylated by a mitogen activated protein kinase 3 (MAPK3) in order to enable the SUB1A-1 for activation of downstream signalling genes to achieve submergence tolerance. Though most of the submergence sensitive rice plants possess SUB1A-2, another allele of SUB1A-1 where 186th serine is replaced by proline and this replacement drastically reduces the ability of MAPK3 to phosphorylate the SUB1A-2 protein leading to poor/no submergence tolerance ability. However, the contrasting ability of SUB1A-1 and SUB1A-2 based on MAPK3 mediated phosphorylation on submergence tolerance is yet to be identified (Lin *et al*., 2023). During this investigation in Hirakud dam reservoir, many rice cultivars having the unusual ability for survival under complete submergence for a period of 25-30 days are available. Though carbohydrates act as the major energy reserves for plant survival, the possibility of up-regulation/ down-regulation of manyother novel stress resistant genes might be involved in different metabolic pathways which needs to be investigated further.

6. Exploitation of rice cultivars for genetic manipulation towards yield enhancement

After the 'Green revolution', the miracle rice variety 'IR-8' was released from International Rice Research Institute, Philippines and subsequently the breeding efforts led to the generation of several IR-8 parented semi-dwarf high yielding rice varieties. The cultivation of such high yielding rice varieties has been highly popularised in most of the rice growing Nations of the World because of tremendous increase in their grain yield potential in comparison to the tall traditional rice cultivars which were grown traditionally by the farmers since time immemorial. Though the high yielding rice cultivars produce more grain yield, they are highly sensitive to most of the abiotic stresses including submergence or water logging and fail to sustain under extreme environmental conditions. But the traditional rice cultivars grown in the Hirakud dam reservoir in the natural ecosystems for ages are being exposed to multitude of both biotic and abiotic stressesand thus acquire a higher degree of resistance towards such extreme environmental stress factors. This resistance trait especially for submergence condition is not only expressed in the phenotype of the plant, but also in the genotype. Several such valuable genes have been identified in the submergence tolerant rice plants by many researchers.

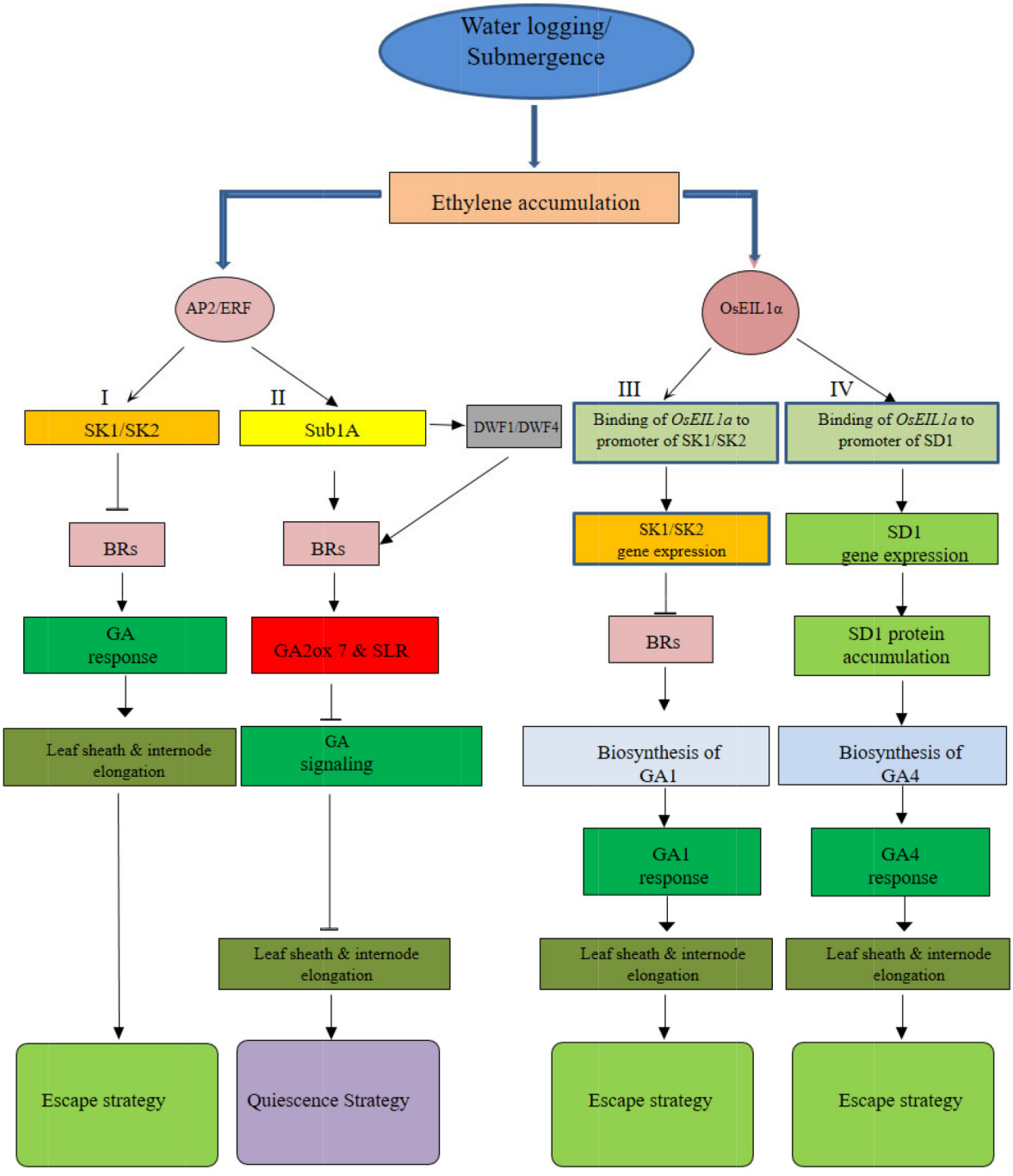

Figure 3: Ethylene mediated submergence tolerance describing one Quiescence and three escape pathways in deep water rice cultivars.

7. Conclusion

Identification and characterisation of stress resistant genes under various abiotic stresses in important crop plants like rice is one of the principal fields of research in present-day context to obtain yield stability under inclement weather conditions. Research efforts in this regard will definitely be of great help inintrogressionof novel stress resistant genes into the high-yielding rice cultivars for production of stress tolerant high-yielding rice genotypes. Though the embankment areas of Hirakud dam reservoir exhibits huge diversity of submergence-tolerant traditional rice varieties, lack of systematic investigation for identification of stress resistant genes precludes their introgression into high yielding rice genotypes for successful cultivation under submerged/flooded environments.Development of

submergence tolerant high-yielding rice genotypes and their successful cultivation would be highly beneficial for exploitation of larger water resources for rice cultivation and increase of farmer's income. Thus, identification of novel submergence tolerant genes from the traditional rice varieties grown in the Hirakud dam reservoir is of high significance for increased rice grain yield and may be largely helpful for providing food security to the ever-increasing human population and also for a solution to World's food problem in future.

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RSM-CCD optimization of factors affecting Chlorophyll extraction from leaves of *Murraya koenigii*: Enhancing the yield of Chlorophyll a

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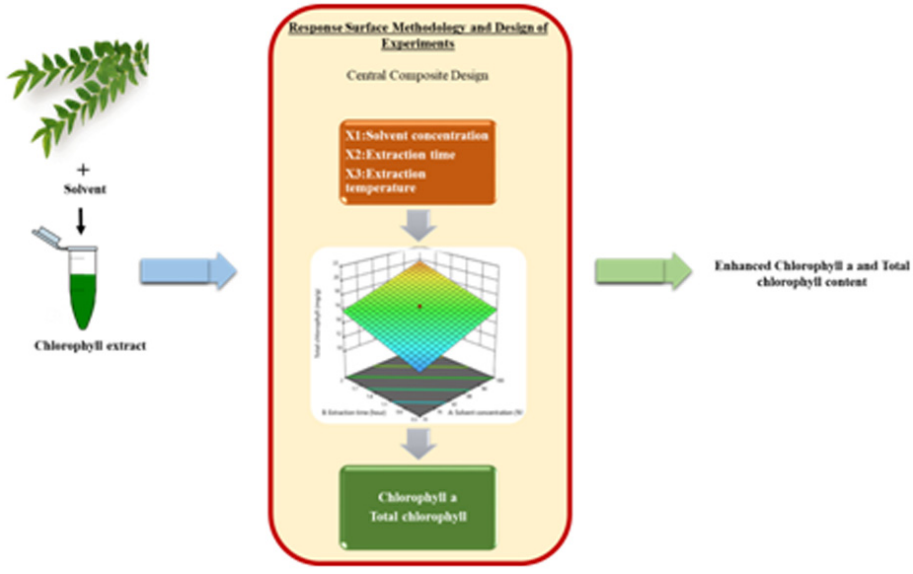
ABSTRACT

Murraya koenigii, or curry leaves (Rutaceae) is indigenous to India and is widely used in Ayurvedic medicine around the world for the treatment and prevention of a wide range of illnesses. Recent research has supported the prospective biological and pharmacological effects of its leaves, such as anticancer, antidiabetic, antioxidant, and anti-inflammatory properties. An improved comprehension of the Chlorophyll (Chl) content of curry leaves will help in relating its therapeutic potential to effective medicine. However, the extraction of pure Chl is challenging due to inefficient extraction and purification techniques. Therefore, we extracted Chl using a solid-liquid extraction method that was dependent on several factors. In this study, the factors affecting the Chl extraction process were optimized by the Response Surface methodology-Central Composite Design (RSM-CCD) approach using Design Expert 11 in relation to solvent concentration, extraction time, and extraction temperature. The responses were obtained after maximizing Chl a and total Chl content. The ideal parameters for the maximum yield of Chl predicted by the software were acetone concentration (100%), extraction time (2 hours), and extraction temperature (50°C). It is anticipated that optimized extraction of Chl will expand its use in various fields.

GRAPHICAL ABSTRACT



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1. Introduction

Humanity has used plants for medical purposes since the dawn of time (Sofowora *et al.*, 2013). Plant extracts are highly valued in many scientific fields, including Ayurvedic medicine, Unani medicine, and others, for their ability to treat various illnesses (Prasath Kumar *et al.*, 2021). Common medicinal herbs that are readily found in our homes are tulsi (*Ocimum tenuiflorum* L.), neem (*Azadirachta indica* L.), and mint (*Mentha arvensis* L.) (Yuan *et al.*, 2016). Among the popular medicinal herbs, *Murraya koenigii* L., commonly known as curry tree/sweet neem, stands out due to its extensive use in Indian culinary and traditional medicines. It is grown wild in forests and under cultivation all over India, Australia, the Andaman Islands, China, Ceylon, Burma, and the Pacific Islands (Abdelwahab and Taha, 2023). It is a member of the Rutaceae family. The leaves are an excellent source of proteins, carbs, and carbazole alkaloids in addition to being high in minerals and vitamins (Nouman *et al.*, 2015). Its possible use as a home medicine for conditions like cancer, diabetes, rheumatism, influenza, and traumatic injuries is described in the literature (Malode *et al.*, 2021). It can also be used to treat piles, inflammation, itching, new cuts, diarrhea, vomiting, bruising, and dropsy (Sarvananda and Umayangani, 2017). Curry leaves are useful in the management of nausea, indigestion, and diarrhea. Additionally, it aids in weight loss and guards against cataract development and early hair graying (Senand Email, 2021).

It has been demonstrated recently that Chloffers therapeutic benefits (Helena *et al.*, 2023). Calculating the amount of Chl present in these plants may demonstrate the significant role that Chl plays in their therapeutic qualities (Solymosi and Mysliwa-Kurdziel, 2016). The following are some of the reported advantages of Chl: It has been observed to aid in tissue growth and healing (Pangestuti and Kim, 2011). Chl is an excellent supplement for smokers as it aids in counteracting the pollutants we breathe in and consume on a daily basis. It transports magnesium to all cells and tissues effectively and aids the blood in delivering oxygen to all tissues (Pangestuti and Kim, 2011). Assimilation and chelation of calcium and other heavy minerals have also been observed to benefit from it. It has demonstrated a strong potential for enhancing the oxygen delivery system by activating red blood cells (Helena *et al.*, 2023). Chl has demonstrated the ability to counteract free radicals, which can cause harm to healthy cells, in conjunction with other vitamins like A, C and E (Hayes and Ferruzzi, 2020). In addition, body odour, urine odour, and foul breath can all be effectively eliminated by Chl. It might make carcinogens less able to attach themselves to DNA in the body's primary organs. The symptoms of calcium oxalate stones may be alleviated by Chl (Mishra *et al.*, 2011). Furthermore, it has some anti-atherogenic properties. Infected wounds can be naturally treated with it. This substance possesses antimutagenic and anticarcinogenic qualities, which could aid in shielding your body from pollutants and lessening the negative effects of medications (Vaòková *et al.*, 2018).

Determining the Chl content of typical curry leaves may aid to a better understanding of their therapeutic uses (Meher *et al.*, 2018). In addition to their added therapeutic value, they can be a cheap and readily available source of Chl. They have no detrimental effects and can be used naturally (Raju *et al.*, 2007). Curry leaves are foods that we might include in our diets. Therefore, this medicinal plant is used for investigation and is quite affordable and widely accessible. Few studies have been conducted on the extraction of Chl from curry leaves (Ahmad and Ramli, 2018). It has been challenging to extract pure Chl from natural resources since the purification processes are more complicated and require greater attention to prevent heat and light damage (Danesi *et al.*, 2004). Numerous studies have also been conducted on the optimization of variables such as solvent type, solvent concentration, solvent/leaf ratio, extraction time, extraction temperature, etc. to produce an adequate quantity of Chl from plant resources (Thao *et al.*, 2022).

Response surface methodology (RSM) is a well-established set of statistical and mathematical procedures used to analyse experimental data using an empirical model (Tran *et al.*, 2019). This was first demonstrated by Box and Wilson, who showed that a reaction to input variables or factors influencing it is connected with fundamental experimental design and analysis. This includes different ways of optimizing the factorial variables for the production of the highest or lowest response value. Factorial methods and ANOVA with more detailed modelling are used to model the response output. The most common method for validating RSM is Central Composite Design (CCD) or Box-Behnken Design (BBD). BBD covers a very small number of design points compared to the axial points of CCD, which increases the number of tests in CCD. Better results for quadratic models are produced by CCD, which encompasses all extreme circumstances. In order to concentrate on the impacts and further sensitize the model, this study's three-level factorial design allows for the summation of additional treatments. In order to maximize the extraction of Chl*a* and total Chl content, this is employed as an enhancement to the current procedures.

This study aims to optimize the factors affecting Chl extraction using the response surface methodology (RSM)

method and Design Expert 11 software to increase the extraction efficiency of Chl a from curry leaves. Moreover, the crucial roles of input factors in the process of Chl extraction were also examined.

2. Materials and methods

2.1. Sample preparation

The leaves of *Murraya koenigii* were collected on the campus of NIT Rourkela. The leaves were rinsed with distilled water three times to avoid contaminants on the leaf surface. They were blotted dry using a paper towel and stored at 4°C for subsequent analysis.

2.2. Solvent extraction

Fresh leaves (2 g) were weighed and cut into small pieces (approximately 9 –15 mm^2). The sample was ground using a mortar for 1 minute using 3 ml of acetone, and the mixture was homogenized using 10 mg of $CaCO_3$ for 2 –4 minutes until a green solution was obtained. Then, using 7 ml of acetone, the mixture was transferred to a beaker and kept for 1 hour at 40 °C. Then, the extract was centrifuged for 15 minutes at 7000 rpm to obtain the supernatant. The supernatant was filtered by Whatman filter paper of pore size (20–25 µm), and the solution obtained was evaporated by a vacuum rotor. The precipitate obtained was dissolved in an appropriate amount of acetone for subsequent experiments (Jinasena *et al.*, 2016; Thao *et al.*, 2022) (Fig. 1).

2.3. Extraction optimization

Optimization of parametric levels was done through comprehensive analysis using RSM-CCD. The complete design of experiments and statistical evaluation were conducted using Design Expert 11 software. Three factors and two responses were used in the design of the experiments in relation to solvent concentration (X1, %), extraction time (X2, hour), and extraction temperatures (X3, ^{0}C), as shown in table 1. Responses recorded were optimized for maximum Chl a (Y1) and total Chl content (Y2). By using CCD, 17 experiments with variable factor ranges were designed, and the responses were fitted to quadratic, linear, tertiary, or 2-fi models. ANOVA was used to assess the best fit model, which was further validated by the p-value, the squared correlation coefficient (R^2) both adjusted and predicted, and the lack of fit. Finally, the optimized responses were built using a statistical model.

Figure 1: Schematic illustration of chlorophyll extraction process.

Table 1

Representation of factors and responses used in response surface design of Chl extraction process.

Response Surface Methodology		
Factors	Low limit	High limit
X1:Solvent concentration	70	100
X2:Extraction time	0.5	2
X3:Extraction temperature	30	60
Responses (mg/g)	Goal	
Chl*a*	Maximize	
Total Chl	Maximize	

2.4. Determination of Chl a and total Chl content

Optical density (OD) was recorded using a spectrophotometer to estimate the amount of Chl present in individual solutions. Spectrophotometric analysis was used to ascertain the amounts of Chl *a* and *b* in acetone extracts.At 645 nm and 663 nm, respectively, the The absorption peaks were measured and the concentrations of Chl *a*, Chl *b* and total Chl were determined (Su *et al.*, 2010).

2.5. Statistical analysis

All the experiments were carried out in triplicate, and the responses were recorded in the form of the mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA, and Bonferroni multiple comparison tests were used for multiple statistical comparisons for experiments involving three or more groups. $P<0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Preliminary screening of Chl extraction

Preliminary screening of the extraction was limited to varying one parameter (solvent concentration, extraction time, and extraction temperature) of the Chl extraction condition. A summary of the initial experimental components has been represented in Table 2.

3.1.1.Effect of solvent concentration on Chl extraction

Acetone of varied concentrations (70 %, 80 %, 90 %, and 100 %) was used for estimating the content of Chl*a* and total Chl from the leaves of *Murraya koenigii*. The highest concentration was recorded at 100 % acetone concentration (i.e., 10.87 mg/g of Chl a and 15.68 mg/g of total Chl) (Table 2).

Table 2

Summary of the initial experimental components.

Parameter	Range	Chla concentration, mg/g	Total Chl concentration, mg/g
Solvent concentration(%)	70	6.21	10.48
	80	7.18	11.37
	90	8.54	13.74
	100	10.87	15.68
Extraction time (hr)	0.5	6.67	9.62
	1	7.19	10.59
	1.5	7.82	10.91
	2	8.43	11.46
Extraction temperature (^{0}C)	30	8.41	13.21
	40	7.48	11.58
	50	11.39	15.34
	60	7.94	11.67

3.1.2.Effect of extraction time on Chl extraction

A constant solvent concentration (acetone 100 %) was set at various extraction times ranging from 0.5-2 hr in order to determine the ideal extraction time. It was observed that the amount of Chl (i.e., 8.43 mg/g of Chl a and 11.46 mg/g of total Chl) obtained was highest at 2 hours (Table 2). A literature study reveals that the diffusion of the particles to be extracted from the raw material into the solution increases with longer extraction times because they will increase the amount of time the raw material and solvent are in contact, boosting extraction efficiency. However,

prolonging the extraction period will reduce the amount of Chl once equilibrium is reached (Abidin *et al.*, 2016).

3.1.3.Effect of extraction temperature on Chl extraction

An acetone concentration of 100 % and an extraction time of 2 hours were the set criteria for an evaluation of extraction temperature. It was noted that Chl (i.e., 11.39 mg/g of Chl*a* and 15.34 mg/g of total Chl) was maximum at 50 °C and minimum (i.e., 8.41 mg/g of Chl a and 13.21 mg/g of total Chl) at 30 °C. However, with increasing temperature, the content decreased (i.e., 7.94 mg/g of Chl *a* and 11.67 mg/g of total Chl) (Table 2). According to the report, the extraction of Chl becomes more effective as the temperature rises to 50 °C. At this temperature (50 °C), the phospholipid layer and hydrocarbon chain that makeup plant cell walls break more easily, allowing the compounds within the leaf to escape the cell wall. Elevated temperatures also contribute to the sample's solubility in the solvent, which in turn accelerates the mass transfer of the solute into the solvent and reduces the extraction time. High temperatures (> 60 °C) can cause Chl breakdown. Thus, it was determined that extraction duration of a maximum of two hours was ideal at 50 °C. Our results were consistent with the reported literature (Ahmad and Ramli, 2018).

3.2. RSM-CCD optimization of Chl extraction

The Central - Composite Design was followed in the execution of the experiments (Table 3). The target function (Y1, Chl*a* and Y2, total Chl content)'s quadratic regression equation with three components [X1, acetone conc (%); X2, time (hr); X3, temperature (^{0}C)]:

$$Y1 = 9.22 + 1.78\ X1 - 0.4402\ X2 + 1.12\ X3 + 0.5249\ X1X2 + 0.2587\ X1X3 + 0.8230\ X3X2 - 2.09\ X12 - 0.31\ X22 - 1.64\ X32 \quad (1)$$

$$Y2 = 5.15 + 0.1355\ X1 + 2.5768\ X2 - 0.0896\ X3 + 0.4587\ X1X2 + 0.2648\ X1X3 + 0.7895\ X3X2 - 1.08\ X12 - 2.41\ X22 - 1.78\ X32 \quad (2)$$

Chl*a* (Y1) and total Chl content (Y2) are shown in Tables 4 and 5, respectively, as the typical summary of the fitting model with response parameters. The model was predicted to be quadratic for both the responses Y1 and Y2, respectively. Many statistical parameters were examined, including the square correlation coefficient, lack of fit, and p-value, in order to verify the fitness of the statistical model. The prospective quadratic model was deemed significant with a 95 % confidence interval when the p-value was less than 0.001. The difference between pure error and relative

Table 3

Experimental configuration and observation of response design in terms of Chl a and total Chl content.

Run	Factor 1X1	Factor 2X2	Factor 3X3	Response 1 Y1	Response 2Y2
1	110.227	1.25	45	11.3	18.97
2	59.7731	1.25	45	6.06607	12.84
3	85	-0.0113446	45	8.01	11.85
4	70	0.5	30	7.34	14.19
5	100	0.5	60	9.42	13.74
6	70	0.5	60	6.41	10.75
7	70	2	30	9.7	16.28
8	85	1.25	70.2269	8.19	14.25
9	85	1.25	45	8.98	15.67
10	100	2	50	13.1	20.99
11	85	1.25	19.7731	11.1	18.14
12	70	2	60	6.38	13.45
13	85	1.25	45	8.98	15.17
14	100	0.5	30	12.67	17.85
15	85	2.51134	45	9.48	19.38
16	100	2	60	10.2	19.54
17	85	1.25	45	9.34	16.49

error from replicated design points is investigated in the "lack of fit tests." The lack of fit value in the current model was greater than the p-value, demonstrating the relevance and dependability of the model. The similarity of the suggested model and the experimental data to one another is indicated by the adjusted and anticipated R^2 values (Raguraman *et al.*, 2018). Better model sensitivity is indicated by an R^2 value that is closer to 1. Our model clearly relates to a low standard deviation and is statistically significant because its R^2 value was greater than 0.9 and the difference between them was less than 0.2.

According to variance analysis results, the regression model for Chl*a* content is statistically significant (Table 4). ANOVA analysis revealed the statistical significance of the regression model for Chl a content ($p < 0.0001$, $R^2 = 0.93$, adjusted $R^2 = 0.91$). Lack of fit was statistically insignificant ($p > 0.05$). This indicates the strong compatibility of the chosen model and suggests that 97 % of the response's values can be explained using the aforementioned suggested model. The model was considered significant when the difference between the adjusted R^2 and projected R^2 was less than 0.2. The ANOVA table showed that the parameter with the most significant effect (high F-value) is solvent concentration, followed by extraction temperature. However, the time period of extraction had the least effect on the response values. A high F-value (10.30) and a low p-value (<0.0001) further demonstrated the relevance of this quadratic model.

In the case of total Chl content, it was evident from the ANOVA table that the factor parameter with the maximum impact (highest F-value) was extraction time ($p<0.0001$), followed by solvent concentration. The response total Chl content showed an R^2 value of 0.95 and an adjusted R^2 value of 0.94, proving the significance of the model (Table 5).

It was observed that with an increase in solvent concentration and extraction time, total Chl yield increased (Fig. 2). These results were in good alignment with the reported literature (Maciej Serda *et al.*, 2013). Maximum extraction was attained at the highest concentration of the solvent. Also, with the increasing time period of extraction, the concentration of Chl increased. This might be due to the increased exposure period of the leaves to the solvent.

The validity of the model has been further examined by analyzing residuals vs. run plots and normal probability. The optimized model of Chl extraction from *Murraya koenegii* displays the correlation between treatment values. Fig. 3 displays the normal probability as well as the residuals vs. run plots. By comparing the experimental and predicted values, it is apparent that the model's treatment values are at the optimal level for the experimental concentration of Chl a, and they also nearly fit the regression line. The same observations also followed for total Chl content. The accuracy of the predicted model is confirmed by the linear probability plot for the residuals. Upon completion of this investigation, we infer that the ideal conditions for maximum Chl extraction are solvent concentration (acetone 100 %), extraction time (2 hours), and extraction temperature (50 °C).

Table 4

Representative model fitting summary of factors elements with response parameter (Y1: Chl a, Y2: Total Chl content) with ANOVA analysis. NS - not significant.

Factors	Y1		Y2	
	Coeff	**F-value**	**Coeff**	**F-value**
Model		55.03		81.68
Intercept	9.22		15.86	
X1	1.78	113.46	1.93	94.58
X2	-0.4402	6.91	2.03	104.62
X3	1.12	44.71	-1.35	45.83
R^2	0.9270		0.9496	
Adjusted R^2	0.9102		0.9380	
F value Lack of fit	10.30 (NS)		1.25 (NS)	
Suggested relation	QUADRATIC		QUADRATIC	

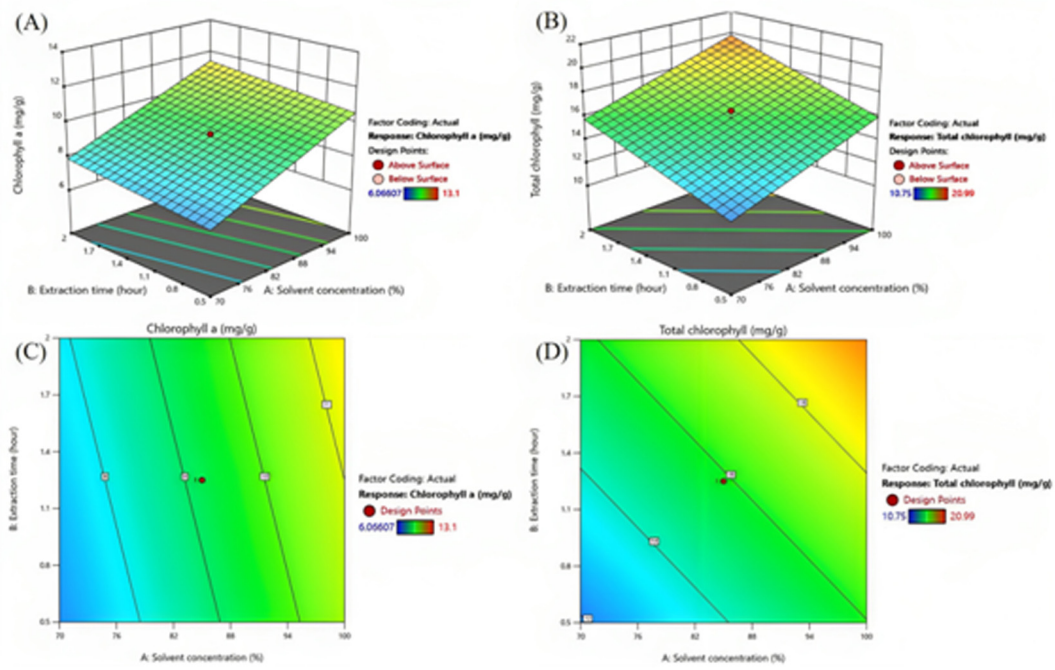

Figure 2: Three dimensional representation of response with surface plots. (A) Chl a (B) Total Chl (C) Contour plot for Chl *a* (D) Contour plot for total Chl.

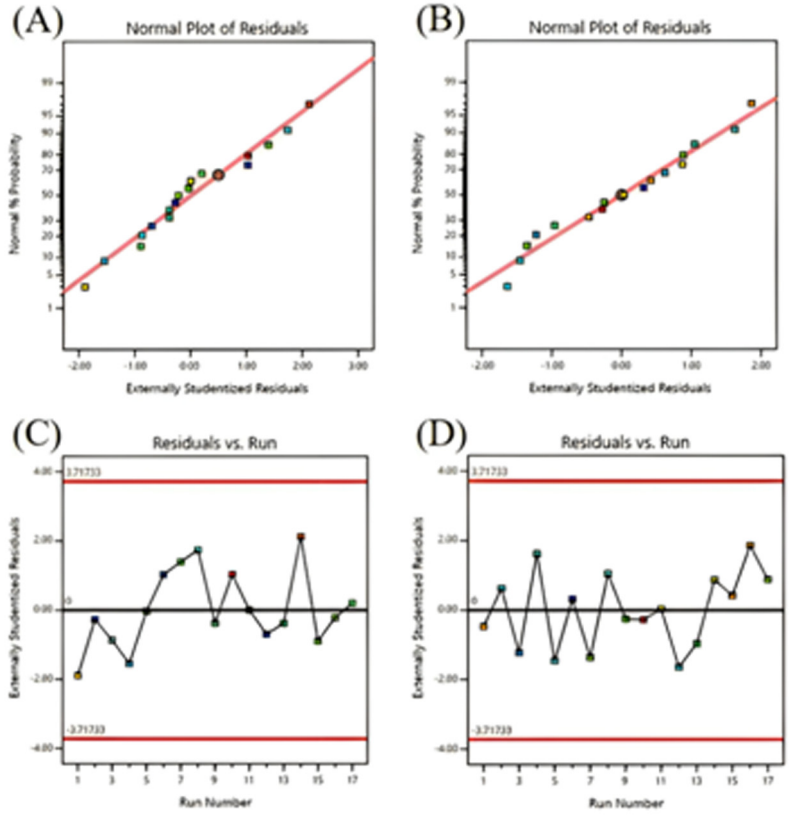

Figure 3: Residual plots (A) normal plot of residuals for Chl a (B) normal plot of residuals for total Chl (C) residual vs run number for Chl a (D) residual vs run number for total Chl.

4. Conclusion

Based on the results, it was determined that the optimal conditions for maximum yield of Chl are acetone concentration (100 %, extraction time (2 hours) and extraction temperature (50 °C). The concentration of Chl a achieved in the experiment was 11.39 mg/g, but the concentration obtained according to the model is 13.1 mg/g under these ideal conditions. This indicates that the optimal model is compatible with 98.7% expectation. The findings support scale-upgradation to extract pure Chl in case further transformation procedures are required for use in functional foods and medications. Briefly, *Murraya koenegii* leaf extract has good medical potential and can be utilized as a natural supply of Chl.

Author Contributions

SM is the first author and has contributed towards the literature survey, performed the formal analysis along with its data curation and writing of the whole manuscript. RL has contributing in performing experiments. BN is the corresponding author. She provided the idea, supervised and validated the entire process from inception to the final submission, and edited the final manuscript.

Conflicts of interest

Authors declare no conflict of interest.

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Sustainable Use of Land Resources for Agricultural Production: A Review

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ABSTRACT

As increasing population and food demand in the current era, we need to focus on new standards of an agricultural system for more production, which is only possible through proper land evaluation. Sustainability refers to the long-run food production system that contribute a welfare social life by providing sufficient food and services to mankind in a way that are socially responsible, environmentally sound and economically efficient and profitable. Sustainable food production systems and implement resilient agricultural practices increase productivity, maintain ecosystems and strengthen capacity for adaptation to climate change, extreme weather, drought and flooding, which progressively improve land and soil quality. Land resource inventory is needed to specify the proper agenda in agricultural system, which is very poorly understood. Land resources information can be helpful to agriculturists before taking up any farming activities in the areas with addressing the issues. The present review highlights the value of the land resources with its application in relation to conservation strategies, site specific crop selection, development of nutrient management system. The climate change mitigation and potential solutions for maximising land productivity with preserving environmental sustainability also highlighted.

1. Introduction

Food production is the basic need of human beings and even all living organisms depend upon nature for their food. Now a days increasing population creates a highfood demand. To that human beings are mostly dependent on the agricultural system for their survival. The COVID-19 during 2019-20, awaked the society that how importance of the livelihood for the life. Soil erosion, ground water pollution, depletion of natural resources, climate change and poor knowledge about the sustainability are the major cause for reducing the agricultural production. India is primarily based upon agriculture and faces a dilemma problem of population growth, poverty, food crisis and land degradation. To mitigate the poverty and environmental pollution, a sustainable agricultural system is required.

In the 21st century, land degradation is a global issue and by year 2050, it may create a serious threat to the environment, agronomic productivity, food security and quality of life (Bhilare, 2013). FAO (2014) identified five interlinked principles toward sustainable food and agriculture with relevance to land resource planning, such as 1) improving efficiency in the use of resources; 2) natural resource conservation; 3) improving rural livelihoods; 4) enhancing resilience and 5) governance.

The current review is highlighted from existing research on sustainable agriculture and land use in which a greater understanding is necessary in sustainable land management in addition to knowledge regarding this. The present research aims to examine the development of economic system in agricultural activity, perceptions and preferences of

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stakeholders, improvement irrigation water use efficiency, development of land and crop management practices that can adopt to the impact of climate change. The present article will be helpful to the researcher, stakeholder and farmers to aware about the sustainability.

Brundtland *et al*. (1987) defined sustainable development is a "Development which meets the need of the present without compromising the ability of future generations to meet their own needs". The Rio Declaration point out the treaties for the sustainable development, which protects and promotes the sustainable use of the planet's biological resources (UNESCO, 1992). In this regard, the Kyoto Protocol committed the nation of the world to reduces greenhouse gasses emission (UNFCCC, 2008). The main objective of the sustainable development is to balance the production of high yields while preserving the environment and wellbeing of local livelihoods (Godfray and Garnett, 2014; Pretty and Bharucha, 2014). Social concerns are required for agricultural production and land conservation by reducing food waste (Kummu *et al*., 2012; Tscharntke *et al*., 2012) and improving food accessibility (Borras and Franco, 2012). Paz *et al*. (2020) stated that the biodiversity and ecosystem services play an integral part for maintaining food supply. With this, it is mentioned that the natural and agricultural land are the key to achieve sustainability and avoid socio-ecological collapse. Mitchell *et al*. (2013) mentioned that the agricultural productivity is strongly dependent on ecosystem services like pollination, nutrient cycling and pest control. They reported about 60% of the population is lost due to the failed crops over time and the advanced technology adopted the intense form of agriculture, which increased food security and resource production. Kuijt and Goring-Morris (2002) described the agricultural production enabled the population growth and allowed the development of complex society with respect to social differentiation and territorial expansion. It also mentioned the agriculture is dependent on the natural environment, and the social life style is declined due to the over-exploitation of resources and poor agricultural land management. The agriculture is responsible for both the rise and fall of society. Barrett *et al*. (2020) interpreted the environmental conservation is caused to threat due to over population and over consumption. The same threat also has been described by Kentor (2001) and Ceballos *et al*. (2017) respectively, which contribute to social instability and environmental degradation. The impact of agricultural intensification on sustainability in the present scenario is an important issue in developing countries foreign demand and conversion of large areas of natural land into intensively cultivated monocultures (Fearnside, 2001; Pengue, 2005; Reboratti, 2010; Soares-Filho *et al*., 2006). They found that the landscape is highly vulnerable to environmental fluctuations, fragmentation, nutrient runoff, contamination of underground water sources and destruction of natural habitats, which practices are detrimental to the healthy environment. And in the other way the agriculture is necessary to feed the population and for which the balance needs to be achieved between the food production and natural land conservation, which can help the population viability in the long run. From this the future work may be derived a greater focus on the links between changes in technology with behavior and its impact on socio-economic dynamics with respect to local environment. By modelling the bi-directional feedbacks between human demography and land use, the misguided or uninformed agricultural land use planning can lead a socio-ecological system to collapse.

Saturday (2018) reviewed and clarified that the restoration of agricultural land is important for sustainability of agriculture and environment. Singh *et al.* (2016) reported that the investment of agriculture in India is grown at the rate of nine percent annually. Niranjana *et al.* (2021) studied on land resource inventory (LRI) for sustainable development in Bisarahalli-1 micro-watershed in the semiarid region of Koppal district, Karnataka and they found the areas suitable for agriculture, horticulture and forestry. It is concluded that the land resource inventory gives information on areas suitable for growing major annual and perennial crops with limitations, which helps in identifying areas that are deficient or sufficient in major and micronutrients. Many researchers have studied the strategies to improve soil quality in different regions (Khillar and Mallik, 2023), which are shownin Table 1.

Intensification and expansion of land use have been started from the beginning of Anthropocene (Ellis and Haff, 2009), which benefitted the livelihood demands for bioenergy, fibre and food (MEA, 2005). The intensification of agriculture is achieved via the application of unlimited high levels of input, such as fertilizers or herbicides, which pollute the environment and down the health of local livelihoods (Kirkhorn and Schenkar, 2001; Tillman, 1999). Kissinger *et al*. (2012) reported about three quarters of the world's forest has been disappeared due to agricultural expansion practices. It influences soil erosion and fertility due to the careless practices (Cunningham *et al*., 2013; Foucher *et al*., 2014). And it is also mentioned that the expansion of agriculture is the second largest global threat to biological diversity conservation (Maxwell *et al*., 2016) because of deforestation. Jose *et al*. (2019) reported about 42% of the world population depends on agriculture for its livelihood, which derives the economy of most developing countries. For the human and civilizational survival, agricultural production is an essential

because of the growing population and food requirement along with income and employment source (Bathaei and Streimikiene, 2023). In the international economy, agriculture plays a crucial part because of about 1.3 billion people (16%) of the global population are employed by which it contributes the global output of 24% (Elawara, 2016). Institutional support, policy changes and behavioral nature are necessary to integrate ecological and societal knowledge towards sustainable agriculture (Nightingale *et al*., 2020). Agricultural practices are incorporated based on the local ecosystem services, consumer needs and the impact of environmental factors (Lluis *et al*., 2020). An awareness of ecological sustainability on agricultural activities including topography, slopes and soil quality play a vital part to assign the value of environmental factors to the crop system, such as the area under cultivation, agricultural productivity, and income earned (Bathaei and Streimikiene, 2023). Sustainable agriculture determines whether farmers are fit or not with respect to technical knowledge and skills (Komlavi, 2019). Out of various perspectives, a farm's prime concerns are soil conditions, water availability, plant growth and nutrient levels (De Corato, 2020). From biophysical perspective, agricultural growth is influenced by soil fertility, climate and pests (Belay, 2022). Production of crops and livestock management practices, structure and viability are also main operation of a farm.

Sustainability is concerned the meeting of national and global food needs with the quality and securing of food supply, transforming technology and improving food distribution systems efficiency and fairness (Kumar *et al*., 2020). The most commonly used definition of sustainable development is "humanity has the power to create a development that meets the needs of the present without impairing the ability of future generation to meet their own needs" (Hansen, 1996). In the sustainable practices, improving soil management with soil quality, crop rotation and water availability benefitted more to the society with increasing yields and creating sustainable environment (Bhilare, 2013). Economic viability is achieved through sustainable agriculture in reducing machinery, chemical fertilizer and pesticide costs. Environment sustainability is achieved through protecting, replacing and maintaining the natural resources such as soil, water, biodiversity that attribute towards conservation aspects ensuring equitable agricultural products, increasing employment and availability of food products locally rather than distant market. Under this prospect, the increasing future demand of agricultural production with balancing nature preservation is a key challenge for future sustainability of land management (Alexandratos and Bruinsma, 2012; Grau *et al*., 2013; Chaplin-Kramer *et al*., 2015).

2. Methodology

Paramanik (2016) used a study for agricultural land use at Darjeeling district by A.H.P. and GIS methodologies (Kumar, 2016). Bozdag *et al*. (2016) conducted a land suitability analysis for Cihanbeyli (Turkey) country based on the methodologies A.H.P. and GIS (Gurkan *et al*., 2016) for sustainability. Bathaei and Streimikiene (2023) studied a systematic scientific review literature on the sustainable agriculture using the tools SALSA followed by PRISMA methodology. It is analyzed about 157 papers in their review study and noted about 101 indicators by analyzing three dimension, such as social, economic and environment. Out of which most recommended indicators for sustainable agriculture are economic (Technology, Market access and price), environment (Farm structure, Pollution and Soil) and Social (Quality of product and Farmers right). The same dimensions also mentioned by Bhilare (2013). In the sustainable practices, Bhilare (2013) mentioned the methodology for the conservation of soil called as till-age namely Strip-till, Ridge till and No till. In addition, it is mentioned that the Grassed waterway and Filter strip have many advantages to the farmers by conserving soil, nutrient and water.

3. Discussion

The world is towards wayward because of pollution, natural calamities, population growth, irregular of season due to the global warming and use of chemical fertilizer with toxic pesticide materials in agricultural system. The aim of future society is to have agriculture improve with social welfare. To achieve this in the current situation of land degradation, the use of advanced technology with pollution free materials is the major concern. In addition to this improving soil fertility, availability of water, drainage, irrigation and erosion control management should be applied for sustainable agriculture. The LRI database helps in preparing optimum land use plans for the micro-watersheds not only in restoring the ecological balance but also in improving the production on a sustainable basis.

The detail idea of sustainability is understanding the basics of agricultural system with attention to biological and physical world to know about the living earth system. For example, understanding of the conservation strategies, land resource management, nutrient cycling, environmental factors, disease and pest control, cropping system which means the selection of crop with respect to soil quality and local environment (Table 1). Agricultural productions contribute to the society's present and long-term food with maintaining ecosystem functions and human development in the society. Sustainable agriculture plays an important

role in the economic and social development along with creating an ecofriendly environment locally. This alternative agricultural system can solve many problems faced by below-poverty-line farmers with ensuring food supply. Sustainability system is a quality of life which provides a healthy, productive and meaningful life for all the communities present and future.

4. Conclusion

The agricultural system interlinks soil, crop and livestock production practices by creating a healthy environment in which life and livelihood depends on. The use of advance technique with organic farming and conservation management which are adopted to local communities using local resources should be followed in a systematic manner.

A proper management of the available land resources is necessary to ensuring food production, biological diversity conservation and quality of life Thus, limited scarce land resources must be used in a socially acceptable eco-friendly way for sustaining life on the earth.

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Table 1:

Different strategies used in different regions to improve agricultural soil quality. Source: Khillar and Mallik (2023).

Strategy	Region	Process	Reference
Litter turnover	Tropics	The rate of organic matter and C supply and nutrient cycling reactivation	Leon and Osorio, 2014
Forestry Plantations	Tropics	Silvo-pastoral system for nutrient cycling	Kohli *et al*. 2008
Woodlot Islets	Degraded drylands	Silvo-pastoral systems in drylands	Helman *et al.* 2014
Soil Carbon Sequestration	Agroecosystems	Optimal management strategies	Berazneva *et al.* 2014
Integrated Nutrient Management	Sub-Saharan Africa	Soil quality management	Diacono and Montemurro, 2010
Nutrient Management for SOC Sequestration	Sub-Tropical Red Soils (China)	Soil carbon buildup	Gong *et al*. 2013
Manuring	Indus Plains	Application of farm manure	Iqbal *et al*. 2014
Residue Retention as Mulch	Mexican Highlands	Improvement of soil structure	Govaerts*et al*. 2006
Regular Organic Inputs	Western Kenya	Nutrient retention and soil structure improvement	Kimetu *et al.* 2008; Moebius-Clune *et al*. 2011
Urban Waste	Mediterranean Europe	Enhancing soil fertility	Rajan *et al.* 2010; Sortino *et al*. 2014
Soil Biological Management	Global soils	Enhance ecosystem services provisioned by SOC pool	Manley *et al.* 2007
Environmental Awareness	U.S.	Promoting technology adoption	Baumgart-Getz *et al.* 2012

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Impact of individual and combined application of tetracycline and streptomycin on rice seed germination plant performance

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ABSTRACT

Rice seeds are recommended to be pre-treated with antibiotics before sowing to eradicate bacterial diseases. A phytotoxicity test was conducted to evaluate the effects of tetracycline (TC) and streptomycin (STR) individually or in combination as Streptocycline (STC) at three different doses (half the recommended dose; 0.5RD, recommended dose; RD and double the recommended dose; 2RD) on rice seed germination and related parameters. The percentage of germination, mean germination time (MGT), vigour index I, vigour index II and shoot length were significantly affected by types of antibiotics and their dosages. However, root length, fresh weight of roots and shoots of rice seedlings was not significantly affected by the antibiotics or their dosages. The germination parameters were significantly affected in combined application as compared to their single applications. In all cases higher doses (2RD) reduced the germination parameters in comparison to RD and 0.5RD treatments. Combined application of TC and STR at higher doses may inhibit the rice germination parameters and early vigour, thereby could pose serious issues in plant establishment in main rice field. The antibiotics did not caused any marked change in the OJIP fluorescence transients of the fully grown rice plants but there was a reduction of fluorescence at all levels. The primary photochemistry and the performance of plants for photosynthetic energy conversion decreased with the increase in the concentrations of antibiotics.

1. Introduction

Rice (*Oryza sativa* L.) is the most important leading food crops and is known as the grain of life and is synonymous with food for Asians. Rice attained its premier position in a balanced diet by virtue of its unique complex carbohydrate, low fat, low salt, no cholesterol, rich source of iron and zinc, high proportion of lysine and B-complex vitamins, especially thiamine, riboflavin and niacin content (Chaudhari *et al.*, 2018). Rice crops are infested by many pathogens causing a huge crop loss affecting the livelihood. The most important of all the bacterial diseases is bacterial leaf blight (BLB). The causal organism of the disease is a gram-negative bacterium, *Xanthomonas oryzae pv. oryzae (Xoo)*. Under favourable conditions, the disease can cause 6-60% of crop loss within a year (Saha *et al.*, 2015). The yield loss depends on severity of infection, type of rice variety, environmental conditions prevailed in the area as well as cultural practices. The disease may show three different types of symptoms such as leaf blight, wilt or Kresek and yellow or pale-yellow leaf.

Management of BLB can be done through the use of resistant cultivars, alteration of cultivation methods, use of bio-control agents, use of natural products or plant extracts and also by the use of chemicals. The Central Insecticide Board and Registration Committee (CIB & RC), Government of India, recommended a mixture of two antibiotics-streptomycin sulphate (90%) and tetracycline hydrochloride (10%), which was registered under the section 9(3) of the Insecticides Act, 1968. The antibiotic can be applied in three ways on rice plants to prevent the disease, viz. seed

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treatment, seedling dip and foliar spray. For seed treatment, the rice seeds are recommended to be soaked in 40 mg/l antibiotic solution for 12 hours at room temperature before sowing.

There are several reports showing the seeds are negatively affected by antibiotics exposure. The effects include delay in seed germination, inhibition of seedling growth, etc in crops other than rice. Seed germination and early growth of *Brassica campestris* was negatively affected with chlorotetracycline and erythromycin application (Cheong *et al*., 2020; Cheong *et al*., 2021). Oxytetracycline and enrofloxacin at concentrations of more than 10 mg/l affected seed germination, seedling growth and root elongation in wheat plants (Li *et al*., 2023). Similar effects have been observed in other crops like lettuce, carrot, cabbage, tomato, alfalfa, cucumber, maize, sorghum, etc. (Bellino *et al*., 2018; Hillis *et al*., 2011; Pan and Chu, 2016; Ghava *et al*., 2015; Wang *et al*., 2017). However, the physiological changes and growth characteristics of rice under antibiotic treatment have not been properly studies.The present study presents the effects of tetracycline (TC), streptomycin (STR) and their mixture, streptocycline (STC) on seed germination, growth characteristics, fluorescence transients and early vigour of rice seedlings.

2. Materials and methods

2.1 Chemicals and seeds

The certified reference material (CRM) of Tetracycline Hydrochloride (TC, purity: 96.7%) and Streptomycin Sulphate (STR, purity: 95%) were purchased from Sigma Aldrich, Merck, India. Taichung Native 1 (TN1) rice seeds were obtained from ICAR- National Rice Research Institute (NRRI) gene bank and stored in sealed paper bags at 5°C until use.

2.2 Germination test

Laboratory evaluation for the effects of TC, STR and STC on rice seed germination was carried out using the filter paper method according to the International Seed Testing Association (ISTA) test protocols (ISTA, 1985). Seeds were soaked in distilled water overnight before the germination experiment. The water-soaked seeds (25) were placed on a filter paper (9 cm diameter) kept in each petri-dish (9 cm diameter). For each antibiotic compound, the filter papers in petri-dishes were treated with 5 mL of antibiotic solution and covered before placing in an incubator maintained at 26± 2ºC and 80±5 % relative humidity. Different doses of TC were 2 mg/L (half the recommended dose; 0.5RD), 4 mg/L (recommended dose; RD) and 8 mg/L (double the recommended dose; 2RD), and of STR were 18 mg/L (0.5RD), 36 mg/L (RD) and 72 mg/L (2RD). In case of STC, combination of TC and STR were used for different doses. All the antibiotic treatments along with control were tested in five replicates each.

2.3 Early growth parameters

At the end of the experiment, seed germination (%), length of root and shoot (cm), total length, fresh weight of root and shoot (mg), and dry weight of root and shoot (mg) were measured. Germination rate was calculated as the number of seeds that germinated per petri dish divided by the total number of seeds taken. Five seedlings were selected for the measurement of length and weight. The seedlings were weighed immediately to obtain the fresh weight to avoid any drying of the seedlings. Root length was measured from the tip of the primary root to the hypocotyls using a standard centimetre scale. Total length was measured from the tip of primary root to the tip of shoot. For the measurement of dry weight of shoot and root, the samples were kept in an oven (at 55/ ±/ 1/ °C for 72/ h) to achieve constant weight.

The vigour index I and II of the seedlings were calculated as suggested by Abdul-Baki and Anderson (1973) through the formulae:

Vigour index I= seedling length x germination percentage

Vigour index II= seedling dry weight x germination percentage

Mean germination time (MGT) is the average time taken for the seeds to germinate, and was calculated as per the formula given by Ellis and Roberts (1981):

MGT= “ (n*d)/ N

Where, n = number of seeds germinated on each day, d = number of days from the beginning of the test and N = total number of seeds germinated at the end of the experiment.

2.4 Chlorophyll a fluorescence

The measurement of chlorophyll *a* fluorescence from the adaxial surface of leaf (fourth from the apex) was made using a plant efficiency analyzer (Handy PEA, Hansatech Instruments, Norfolk, UK) following the method described by Chhotaray *et al.* (2014). The fluorescence parameters, viz., variable fluorescence (Fv), 300 µs relative variable fluorescence (V_K), 2 ms relative variable fluorescence (V_J), net rate of PS II closure (M_0), quantum yield of primary photochemistry (jP_0), rate of trapped exciton movement beyond Q_A (y_0), quantum yield of electron transport (jE_0), quantum yield of energy dissipation (jD_0), effective antenna

size of active RC (ABS/RC) and performance index of primary photochemistry (PI_j) were calculated using the fluorescence equations of Force et al. (2003) and Stirbet and Govindjee (2011). The chlorophyll *a* (chl *a*) fluorescence was measured from the fully grown (45 days old) potted rice plants. After spray application of the antibiotics, the plants were placed in green house for 7 days at ambient temperature and the fluorescence responses of the plants were taken as a measure of their photosynthetic performance.

2.5 Statistical analysis

The effect of antibiotics, and their doses on seed germination parameters was analysed by using two-way analysis of variance (ANOVA) using SPSS software (IBM Corporation, 2016). The treatments were compared based on by Tukey's HSD (Honestly Significant Difference) at P = 0.05. Percent seed germination data was arcsine transformed before analysis.

3. Results

The germination percentage, mean germination time (MGT), Vigour index I, Vigour index II and other parameters are presented in Table 1. Germination was noticed after 12 h of incubation. Germination percentage (94.6%) was found highest in TC at recommended dose, where as in control the germination percentage was 85.6%, indicating the enhancement of germination by the antibiotics Such enhancement was found significantly altered by type of antibiotics ($F_{2,44}$=12.55) and antibiotic doses ($F_{3,44}$=13.63) (Table 2). Individual application of antibiotics improved the germination percentage as compared to control. The gain in germination percentage was at par with RD and 0.5RD doses of TC and STR. Higher doses (2RD), however, reduced the germination in comparison to RD and 0.5RD treatments. The germination percentage was the least at any comparable dote of STC. Effect of TC and STR; and 0.5RD and RD on seed germination did not have any statistical differences.

The mean germination time (MGT) followed the similar trend as that of germination percentage and ranged from 2.16-2.56 days. It was found that germination efficiency was significantly affected by type of antibiotics ($F_{2,44}$= 7.04) and antibiotic doses ($F_{3,44}$= 6.54). STC was found to increase the mean germination time as compared to TC and STR. Among the doses, 2RD was found to negatively affect MGTas compared to other doses and the untreated control.

The antibiotics type ($F_{2,44}$= 4.12) and doses ($F_{3,44}$= 7.57) caused marked changes on the shoot length of rice seedlings. In comparison to RD and 0.5RD, the 2RD treatment shortened the shoot length thus affecting its growth. The effects of the lower doses were, however, insignificant. When TC and STR were applied together, the shoot length was more affected in comparison to individual applications. However, root length, fresh weight of roots and shoots of rice seedlings was not significantly affected by the antibiotics or their doses applied at 0.5RD but there was significant increase in the toxicity with increase in the concentration. At 2RD, the growth parameters were significantly affected as compared to other treatment doses as well as to the control. Antibiotic treatments had also an impact on the dry weight of shoot and root of rice seedlings. Antibiotic dosages had a substantial impact on shoot dry weight ($F_{3,44}$= 5.94) resulting in dose dependent inhibition. In comparison to 2RD, there was a greater amount of shoot dry weight in the 0.5RD and RD dosages but at all treated doses there was more or less a reduction in the dry weight of the shoot. The similar trend was also observed in the root dry weight ($F_{3,44}$= 5.67).

The types of antibiotics and their dosages had a significant impact on both the vigour indices. Antibiotic type (F2,44= 8.79) and dosage (F3,44= 6.98) had a significant impact on Vigour index-I. Vigour index-II was also significantly affected both by antibiotic type (F2,44= 4.40) and antibiotics doses (F3,44= 10.11)(Table 2). Among the antibiotics applied, STC caused the highest decrease of the vigour of rice seedlings in comparison to TC and STR. However, there was no significant difference between the two antibiotics with regard to the reduction of vigour index. Never the less, vigour indices decreased with increase of antibiotic dose in each treatment. In contrast to the 0.5RD and RD treatments, the 2RD dosage reduced the vigour of rice seedlings in all cases. The effects of the 0.5RD, RD, and control did not differ significantly from one another in case of separate application of the antibiotics but the combined application doses were always inhibitory.

We observed no significant change in the shape of the transients was noticed with antibiotics treatment but there was a decrease in FM without any change in OJIP rise. Such decrease was insignificant with TC treatment at 0.5 RD and RD but significant with STR treatment ? RD and at all doses of STC (Fig. 1). Significant variation in the fluorescence transients at all inflections was not seen with TC treatment up to RD level but at 2 RD the decrease in the fluorescence rise was observed in all inflections. With STR and STC treatments there was variation in the intensity of rise at the peak as well as in intermediate inflections.

Table 1

Effect of different antibiotics and their doses on rice seed germination parameters

Seed germination parameters	Control	TC			STR			STC		
		0.5 RD	RD	2 RD	0.5 RD	RD	2 RD	0.5 RD	RD	2RD
Germination percentage (%)	92.6 ± 1.67	92.4 ± 2.88	94.6 ± 2.96	87.8 ± 3.63	93.2 ±1.78	92.8 ±1.92	88.8 ± 4.14	89.0 ±1.58	84.6 ±5.17	85.0 ± 3.31
Germination time (days)	2.18 ± 0.11	2.18 ±0.02	2.19 ±0.09	2.27 ±0.10	2.22 ±0.11	2.16 ±0.07	2.22 ±0.03	2.21 ±0.18	2.33 ±0.06	2.56 ±0.19
Shoot length (cm)	4.86 ±0.45	4.79 ±0.20	4.60 ±0.14	4.55 ±0.36	4.86 ±0.45	4.31 ±0.50	4.16 ±0.44	4.42 ±0.44	4.11 ±0.69	3.83 ±0.52
Root length (cm)	3.62 ±0.48	3.70 ±0.41	3.40 ±0.42	3.31 ±0.24	3.98 ±0.35	3.62 ±0.48	3.62 ±0.40	3.70 ±0.45	3.45 ±0.26	3.19 ±0.46
Shoot fr wt (mg)	4.85 ±0.49	5.11 ±0.59	5.10 ±0.54	5.02 ±0.50	5.12 ±0.38	4.90 ±0.43	4.85 ±0.49	5.03 ±0.36	4.78 ±0.87	4.71 ±0.50
Root fr wt (mg)	1.61 ±0.15	1.70 ±0.12	1.67 ±0.16	1.61 ±0.15	1.70 ±0.19	1.67 ±0.11	1.63 ±0.14	1.69 ±0.18	1.59 ±0.29	1.56 ±0.16
Shoot dry wt (mg)	2.27 ±0.06	2.35 ±0.40	2.28 ±0.10	1.98 ±0.36	2.25 ±0.22	2.17 ±0.36	2.00 ±0.68	2.19 ±0.52	2.13 ±0.59	2.04 ±0.07
Root dry wt (mg)	1.18 ±0.03	1.23 ±0.11	1.17 ±0.19	0.99 ±0.18	1.32 ±0.20	1.24 ±0.12	1.09 ±0.26	1.06 ±0.29	1.05 ±0.06	0.99 ±0.03
Vigour index I	8.26 ±0.57	7.84 ±0.47	7.58 ±0.67	6.90 ±0.73	8.23 ±0.72	7.35 ±0.75	6.91 ±0.48	7.22 ±0.64	6.98 ±0.99	5.97 ±0.50
Vigour index II	2.56 ±0.09	3.30 ±0.40	3.26 ±0.34	2.60 ±0.39	3.56 ±0.20	3.44 ±0.47	2.74 ±0.16	2.89 ±0.73	2.70 ±0.76	2.57 ±0.11

Table 2

The coefficients of two factor ANOVA as a measure of the effects of antibiotics and doses.

Parameter	Antibiotics	Doses	Combination
Seed germination	12.55*	13.63*	2.95*
Shoot length	4.12*	7.57*	0.76
Root length	2.13	2.01	1.05
Shoot fr. Wt.	0.62	0.72	0.25
Root fr. Wt.	0.64	0.36	0.45
Shoot dry wtr.	1.48	5.94*	0.64
Root dry wt.	1.54	3.67*	2.63*
Mean germination time	7.037	6.535	2.742
Vigour index I	8.79*	6.98*	1.00
Vigour index II	4.40*	10.11*	1.24

Note: * significant at P≤0.05.

Even though some variations in the magnitude of V_K was observed with the treatments, there was no specific trend in the relative fluorescence at this inflection. Same trend was seen in the level of V_J with TC and STR treatment. However, with STC treatment, V_J was significantly high at 2RD (Table 3). In all treatment a decrease in φP_0 was observed, which was significant 2RD with TC or STR treatment but at ≥ RD with STC treatment. Similar trend was also observed for the exciton (ψ_0) and electron movement beyond Q_A (φE_0). Dissipation (φD_0), on the other hand, increased with increase in the antibiotic concentration. Such increase was significant at 2RD in all cases. There was no marked change in M_0 with TC treatment but a decreasing trend was observed with STR and STC treatments. ABS/RC did not vary significantly with STR or TC treatment but increased significantly with STC treatment. PI_φ showed continuous decrease with increase in the concentration of each antibiotic.

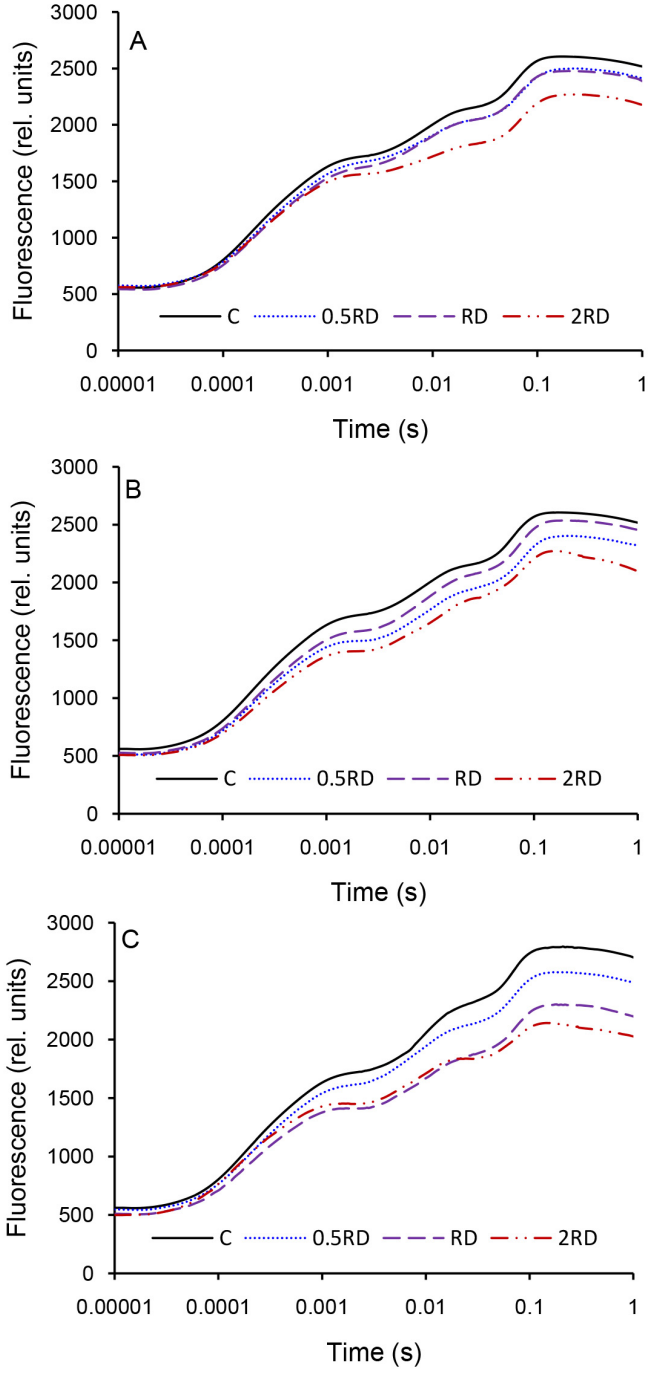

Figure 1: The OJIP fluorescence transients of fully grown rice plants on treatment with (A) tetracycline, (B) streptomycin and (C) streptocyclin. The measurement of the transients was made 7 days after treatment.

Table 3

The bioenergetic attributes of OJIP transients of fully grown rice plants, 7 days after foliar application of antibiotics.

Appl. Conc.	V_K	V_J	jp_0	y_0	jE_0	jD_0	M_0	Abs/RC	PIj
				Tetracyclin					
0	0.336^{ab} ±0.012	0.566^{ab} ±0.021	0.785^{a} ±0.045	0.434^{a} ±0.021	0.341^{a} ±0.013	0.215^{b} ±0.008	1.305^{a} ±0.062	1.867^{a} ±0.063	3.647^{a} ±0.117
0.5RD	0.320^{b} ±0.011	0.568^{ab} ±0.033	0.771^{a} ±0.036	0.432^{a} ±0.011	0.333^{a} ±0.017	0.229^{b} ±0.008	1.281^{a} ±0.049	1.739^{a} ±0.091	3.362^{a} ±0.128
RD	0.322^{b} ±0.016	0.556^{b} ±0.035	0.781^{a} ±0.028	0.444^{a} ±0.009	0.347^{a} ±0.009	0.219^{b} ±0.009	1.286^{a} ±0.058	1.807^{a} ±0.102	3.373^{a} ±0.104
2RD	0.349^{a} ±0.018	0.584^{a} ±0.022	0.742^{b} ±0.018	0.401^{b} ±0.018	0.313^{b} ±0.021	0.258^{a} ±0.007	1.397^{a} ±0.063	1.798^{a} ±0.104	3.036^{b} ±0.113
				Streptomycin					
0	0.326^{a} ±0.009	0.525^{a} ±0.022	0.785^{a} ±0.033	0.444^{a} ±0.017	0.351^{a} ±0.016	0.215^{b} ±0.009	1.345^{b} ±0.066	1.867^{a} ±0.092	3.647^{a} ±0.102
0.5RD	0.315^{a} ±0.016	0.520^{a} ±0.019	0.786^{a} ±0.028	0.440^{a} ±0.014	0.368^{a} ±0.017	0.214^{b} ±-.008	1.262^{b} ±0.048	1.909^{a} ±0.068	3.677^{a} ±0.167
RD	0.308^{a} ±0.011	0.524^{a} ±0.015	0.783^{a} ±0.042	0.446^{a} ±0.021	0.327^{b} ±0.022	0.207^{b} ±0.011	1.331^{b} ±0.029	1.863^{a} ±0.071	3.629^{a} ±0.182
2RD	0.328^{a} ±0.019	0.538^{a} ±0.017	0.746^{b} ±0.023	0.408^{b} ±0.023	0.308^{c} ±0.014	0.254^{a} ±0.014	1.582^{a} ±0.072	2.066^{a} ±0.038	3.257^{b} ±0.104
				Streptocyclin					
0	0.308^{b} ±0.014	0.518^{b} ±0.017	0.799^{a} ±0.042	0.482^{a} ±0.044	0.386^{a} ±0.011	0.201^{b} ±0.008	1.231^{b} ±0.052	1.901^{b} ±0.065	3.986^{a} ±0.102
0.5RD	0.311^{b} ±0.011	0.526^{b} ±0.016	0.789^{ab} ±0.023	0.474^{a} ±0.021	0.374^{a} ±0.019	0.211^{b} ±0.009	1.244^{b} ±0.038	1.867^{b} ±0.038	3.736^{b} ±0.143
RD	0.318^{b} ±0.016	0.504^{b} ±0.028	0.779^{b} ±0.029	0.486^{a} ±0.039	0.326^{b} ±0.009	0.221^{ab} ±0.007	1.272^{b} ±0.043	1.966^{ab} ±0.093	3.529^{c} ±0.214
2RD	0.402^{a} ±0.022	0.582^{a} ±0.027	0.733^{c} ±0.034	0.418^{b} ±0.026	0.319^{b} ±0.012	0.267^{a} ±0.011	1.608^{a} ±0.038	2.111^{a} ±0.087	3.227^{d} ±0.113

Note: Means superscripted by same letter(s) are not significantly different at p=0.05 tested through LSD.

4. Discussion

Rice seeds were chosen for this experiment due to its agronomic significance. When the antibiotics were applied singly, the germination percentage was found to increase in contrast to their combined application (20-80 mg/L) which decreased the germination percentage. With the increase in antibiotic doses, the germination was negatively affected. Generally, xenobiotics including antibiotics at low doses did not have effect on seed germination, but could affect significantly on radicle elongation stage (Luo *et al.,* 2019). Hard seed coats protect the embryo of seeds (Geneve *et al.,* 2018). A study by deRopp (1948) suggests that STR is a general inhibitor of the growth of embryonic tissues. This might be the reason that higher concentration of STR negatively affected seed germination of rice in our study. TC group antibiotics (TC, CTC, OTC etc.) have been reported to pose negligible or no effects on seed germination in several previous literatures which used TCs at 1-100 mg/L concentrations (Hillis *et al*., 2011; Yang *et al*., 2010), though however, the germination was affected at higher doses.

Compared to germination percentage, the mean germination over time could be a better indicator to test the effect of antibiotics on seed germination. STC was found to delay germination as compared to TC and STR but insignificant delaying was also reported on treatment with TC and STR. Among the doses, 2RD was found to negatively

affect MGT as compared to RD, 0.5RD and control in all treatments. It supports previous observations that antibiotics, which are active against gram negative microorganisms, delay the seed germination process (Goss, 1962). By comparing the germination and MGT data, it can be concluded that antibiotics do not decrease the germination percentage but delay the process thus causing a slowing down the biochemical events during germination. Also, the seeds for which germination was delayed are likely to face competitive pressure for further growth in terms of root and shoot development.

After seed germination the correct development of shoots and roots is critical for plant establishment and survival. Some antibiotics may have lesser toxic effects on seed germination, but pose direct negative effects on emerging roots and shoots (An *et al.*, 2009). In our study shoot development were found significantly affected by antibiotic treatments. Earlier it was reported that TC at 0.01 mg/L stimulated the shoot growth of lettuce, carrot, cucumber and tomato (Pan and Chu, 2016). The authors also reported a declined trend for shoot elongation on increasing the concentration of TC. In our study, however, a decrease in shoot and root growth as well as their biomass were observed at RD and at 2RD of individual antibiotic as well as in the combination, though insignificant stimulation was noticed at 0.5 RD. Toxic effects on root elongation at high TC dosages have been reported with by other authors in other edible crops (Pan and Chu 2016; Liu *et al.*; 2009).The significant effect of antibiotics on total length of seedlings and total dry weight of seedlings is reflected as Vigour indices confirming that vigour index can be a reliable indicator of the toxicity assessment of antibiotics.

No remarkable difference in the shape of OJIP rise with treatment indicated that the antibiotics moreor less equally affected the fluorescence rise at all levels. In most cases a good value of φP_0 (>0.7) was observed that the chemicals did not cause severe inhibition of photosynthetic performance as seen in other stressors (Shasmita *et al.,* 2020; Strasser *et al.,* 2004; Stirbet and Govindjee, 2011). Nevertheless there was a rising trend of V_J, especially with STC treatment indicating that the photosynthetic performance of plants was affected with treatment (Qiu *et al.,* 2004). Similar change was also reported with respect to ψ_0 and φE_0 indicating that not only the primary photochemistry but and the electron movements from PS II is affected by the treatments, especially at higher dose (2RD). A good level of heat dissipation was observed at higher doses of antibiotic treatment showing that the plants released the excess absorbed energy as a protection mechanism against light stress. This is because of the fact that the proportion of closed photosystems increased with antibiotic doses, which was not significant, this resulted in lowering of P fluorescence as seen from the transients. Consequently the absorption area per reaction centre also increased. No significant change in the magnitude of V_K is an indicator of lack of the effect of treatment on donor side of PS II (Strasser, 1997; Lazar, 2003).

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Analysis of genetic diversity among fluorescent Pseudomonads isolated from groundnut rhizosphere

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ABSTRACT

Fluorescent Pseudomonads as plant Growth Promoting Rhizobacteria (PGPR) has played a significant role in plant development with decrease in the severity of several fungal diseases. The present research work focus on characterization of genetic diversity of isolated 24 fluorescent Pseudomonads isolated from different rhizosphere of the groundnut in the Rayalaseema region of Andhra Pradesh. Genetic diversity was analyzed by RAPD markers and among the studied 12 primers; eight primers produced 100% polymorphic bands while the OPC12 primer showed a monomorphic band. A total number of 87 amplified bands were identified, of which 80 polymorphic bands were noted and the PIC value ranged from 0.12 to 0.36 for polymorphic markers.The dendrogram analysis divided the isolates into two major and two minor clusters. The knowledge gathered here about the genetic diversity of fluorescent *Pseudomonads* associated with the ground soil rhizosphere helps to understand their role and potential use in sustainable agriculture.

1. Introduction

The genus *Pseudomonas* comprises a group of omnipresent microbes found in the various ecological habitats, such as soil, water, sediments, fungi, animals, plants and humans surfaces possibly just because of their easy nutritional necessities (Brown *et al*., 2012; Rainey *et al*., 1993; Vela *et al*., 2006; Scales *et al*., 2015; Jogi *et al*., 2020). Fluorescent *Pseudomonas* spp. can be visually distinguished from other *Pseudomonas* by their capability to synthesize a yellow-green fluorescent compound (Jogi *et al*., 2020). Fluorescent Pseudomonads strain can be isolated from plant surroundings and are usually stated as plant growth-promoting rhizobacteria (PGPR) (Jogi *et al*., 2020). These Fluorescent Pseudomonads PGPR species have a biotechnological interest because of their capability to influence plant hormonal balance (Kang *et al*., 2006) and improve plant vigor by decreasing the effects of plant-pathogens.

The Random amplified polymorphic DNA (RAPD) is widely used for the SCAR molecular markers development, diversity assessment, and identifying markers linked with traits of interest in microbes and plants. Primers are designed randomly about 10 bp and the technique is used in organisms where the DNA sequence is unknown (Williams *et al*., 1990). Irrespective of the source or age of the organism, the RAPD patterns are fairly accurate and exceptionally helpful for germplasm characterization, estimation of diversity, and genetic resource conservation programs (Welsh and McClelland, 1990). The RAPD markers have been utilized successfully to estimate the genetic variability of fluorescent Pseudomonads (Patel *et al*., 2018; Megha *et al*., 2007).

The genetic diversity assessment of several plant species was reported very frequently by several researchers. The RAPD molecular marker technology facilitates the genetic characterization of *Ocimum* (Patel *et al*., 2015), *Changiums myrnioides* (Fu *et al*., 2003), coffee species (Mishra *et al*., 2014), and simultaneous identification of *T.*

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cordifolia, E. officinalis and *T. terestris* (Shinde, 2007). Molecular and genetic analysis of *Urginea indica, Rauvolfia serpentine,* and *Rauvolfia tetra phyla* L., using RAPD markers has been studied (Padmalatha and Prasad, 2006 & 2007; Ruan *et al*., 2008). The RAPD technique has been generally used for genetic assessment of wild plants (Khasa and Dancik, 1996; Manica-Cattani *et al*., 2009).

The RAPD molecular markers are simple and resourceful and have been employed toassess the genetic variation at the molecular level in fluorescent Pseudomonads. In general, establishing the fluorescent Pseudomonads genetic diversity from the groundnut crop rhizospheres through genetic markers is a critical step in determining the frequency of distribution of these isolates in various locations of the Rayalaseema region. To keep these in view the genetic variation among the 24 isolates of fluorescent *Pseudomonads* using selected 12 primers RAPD were characterized.

2. Materials and methods

2.1. Bacterial strains

The fluorescent Pseudomonads were isolated from soil samples of groundnut rhizosphere at the Rayalaseema region of Andhra Pradesh, India (Jogi *et al*., 2020). These isolates were maintained at glycerol (50%) stocks at -80 0C.

2.2. Genomic DNA isolation

Genomic DNA was isolated from fluorescent Pseudomonads following the protocol described by Ausubel *et al.* (1992) with few modifications.

2.3. RAPD-PCR

Twelve random primers, namely OPA-01, OPA-11, OPA-17, OPD-01, OPD-02, OPD-03, OPE-02, OPE-04, OPF-10, OPF-11, OPF-14, and OPC12 (Operon technologies Inc.), were evaluated to construct the dendrogram. PCR mix was freshly prepared by mixing 10 X assay buffer (2.5µl), 2.0mM $MgCl_2$ (2.0µl), 10mM dNTPs (0.5µl), 0.5 units of Taq DNA polymerase (0.2µl), 0.6 ìm primer (2µl) and sterile milli Q water (15.8µl) in 0.2 ml PCR tubes. To this 23µl of PCR mixture, 2µl of genomic DNA (30µg) was added. Amplification was carried out by following the PCR program; 5min of initial denaturation at 94°C, followed by 40 cycles of denaturation at 94°C for 1min, annealing at 37°C for 2min, extension at 72°C for 2min and final extension step at 72°C for 10min. The amplified PCR products were separated on 1.5% agarose gel electrophoresis with 1X TBE buffer and the amplified products were visualized and the RAPD-banding pattern was documented using Gel documentation instrument.

2.4. Data Analysis

The RAPD-PCR and gel electrophoresis experiments were repeated at least three times to verify the banding pattern and certain reproducible bands on the gels were evaluated for data analysis. A score of "1" was given for the presence and "0" for the absence of bands. All the reproducible bands of 12 RAPD gels were scored for the corresponding band as presence (1) and absence (0) in all 24 Fluorescent pseudomonads samples. The binary data consisting of "1" and "0" generated for all alleles or bandswereevaluated for the Jaccard's similarity coefficient values and genetic similarity using unweighted pair group arithmetic mean (UPGMA) program using NTSYSpc 2.02j software. The Polymorphism Information Content (PIC) value was calculated following the formula; PIC = 2fi(1-fi), Where, fi is the frequency of the amplified allele (band present) and (1-fi) is the frequency of the null allele (band absent) (Roldán-Ruiz *et al*., 2000; Soengas *et al*., 2006).

3. Results

A total of 24 fluorescent Pseudomonads were isolated from ground cultivating areas of Andhara Pradesh (Jogi *et al*., 2020). All of the isolates were screened against *S. rolfsii* (data not shown) and these effective isolates have been used forgenetic diversity studies.

3.1. RAPD-PCR analysis

Genomic DNA of 24 PGPR bacterial isolates was successfully amplified with 12 RAPD oligonucleotide primers. The amplified products were analyzed to detect polymorphism and genetic diversity among the fluorescent Pseudomonads. The number of amplified products obtained was specific to each primer, and it ranged from 1 to 12 (Table 1). A total of 87 alleles were scored, of which 80 (91.95%) were polymorphic (Table 1). Out of 12 oligonucleotide primers, 8 primers viz. OPA-01, OPA-11, OPD-01, OPD-02, OPE-04, OPF-10, OPF-11, and OPF-14 produced 100% polymorphic bands,while OPA-17, OPD-03, and OPE-02 primers gave both mono and polymorphic band patterns and one primer (OPC-12) produce monomorphic banding pattern (Table 1).The amplification patterns revealed a high level of polymorphism and multiple DNA amplification products with 5 to 12 alleles were noted, except OPC-12 (Table 1). The maximum number of alleles was observed in OPD-1 and OPF-10 (Table 1). The OPA-01 and OPA-17primers produced 9 alleles, OPA-11 primer produced 8 alleles, whereas OPD-03, OPE-02, OPE0-4, OPF-11, and OPF-14 primers produced only 5 alleles (Table 1).The polymorphism information content (PIC) was calculated for each primer and it's ranged from 0.5 to 0.36, whereas OPC-

12 gives monomorphic bands and had a PIC value of zero (Table 1). The highest PIC values were observed in OPD-01 and OPF11 followed by OPA-01 and OPA11 (Table 1).

The dendrogram revealed two major clusters and two minor clusters and the major clusters were subdivided into different sub-clusters (Figure 1). The Jaccard's similarity coefficient matrix was examined for highest and lowest similarity coefficient values for each fluorescent Pseudomonad strains (Table 2). Among them, PGY1, PGY5, PGY3, PGY4, PGY6, PGY13, PGY17, and PGY18 strains belonged to the major cluster-I (Figure 1). The PGY5 strains showed the highest similarity coefficient with PGY1, PGY3, PGY4, and PGY6 stains (Table 2), hence all these strains were associated in sub-cluster-Iof the cluster-I (Figure 1). The PGY13 and PGY18 showed the highest similarity coefficient with PGY17, and the PGY17 showed the highest similarity coefficient with PGY18, hence these three were included in thesub-cluster-IIof cluster-I (Table 2 and Figure 1). The PGY5 had the second-highest similarity coefficient with PGY13, PGY17, and PGY18 strains; therefore all these eight strains were included in cluster-I. The PGY5 strain forms a link between the sub-clusters-I and sub-cluster-II; hence these two sub-clustersfall in the major cluster-I (Table 2 and Figure 1).

Another major cluster-II consisting of PGY7, PGY9, PGY8, PGY11, PGY14, PGY16, PGY15, PGY12, PGY19, PGY24, and PGY20 strains, and these were classified into three sub-clusters (Figure 1). The PGY7, PGY9, PGY8, and PGY11 formsub-cluster-Iof the cluster-II (Figure 1). The analysis of similarity coefficient values suggested that the PGY7 had the highest similarity coefficient with PGY9 and the PGY8 had the highest similarity coefficient with PGY11 (Table 2). The PGY9 had the second-highest similarity coefficient with PGY11 (Table 1). Due to these similarity coefficient values, these four strains fall in sub-cluster-I (Figure 1). The PGY14 had the highest similarity coefficient with PGY16, while PGY 15 and PGY16 showed the highest similarity coefficient with PGY14; hence these three strains were classified as sub-cluster-II of the major cluster-II (Figure 1). The strain PGY24 had the highest similarity coefficient value with PGY19 and the PGY12 and PGY19 had the second-highest similarity coefficient values with PGY24 (Figure 1). Consequently, these three PGY12, PGY19, and PGY24 form the sub-cluster-III of the major cluster-II (Figure 1).

The other isolates PGY21 & PGY22 and PGY10 & PGY23 are classified into two small clusters (Figure 1). The PGY21 and PGY22 strains showed the highest similarity coefficient values with each other and belong to a separate minor cluster-III of the dendrogram (Figure 2). The PGY23 strain showed the lowest similarity coefficient value with PGY2, PGY4, PGY5, PGY11, PGY12, PGY17, and PGY24; hence the PGY23 strain grouping away from all other clusters (Table 1 and Figure 1). The PGY1 to PGY6 samples were collected from the Anantapur district were placed in sub-cluster-I of cluster-I and have a similarity coefficient value of more than 0.75 (Figure 1). Three of the PGY13, PGY17, and PGY18 isolates collected from the Kadapa district were categorized in the sub-cluster-II of the cluster-I (Figure 1). Out of 12 isolates collected from the Anantapur and Kadapa districts of the Rayalaseema region, nine isolates were grouped into thecluster-I of the dendrogram. Whereas cluster-II was formed with three sub-clusters mainly samples collected from three districts (Figure 1). The isolates collected from Kurnool (PGY7, PGYPGY9, PGY8, and PGY11) and Kadapa (PGY14, PGY16, and PGY15) were categorized into two sub-clusters of the dendrogram (Figure 1). The two isolates (PGY19 and PGY24) isolated from the Chittoor district were placed in sub-cluster-III of thecluster-II (Figure 1).

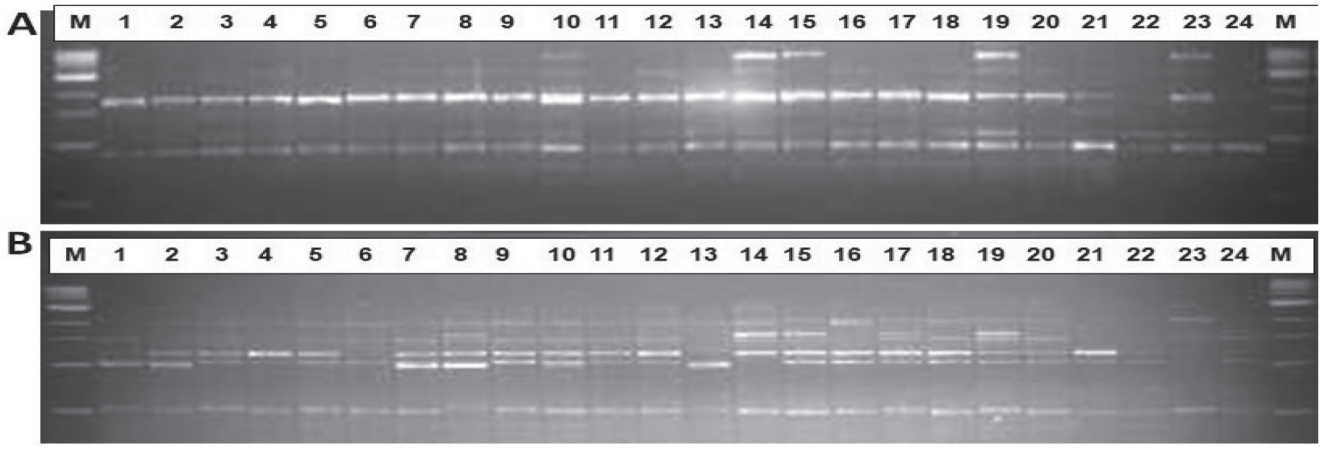

Figure 1: Random Amplified Polymorphic DNA (RAPD) amplification profile of 24 isolated fluorescent Pseudomonadsstrains with primer OPA-3 (A) and OPA-10 (B); M: 1 kb DNA marker.

Table1

List of primer and degree of polymorphism of RAPD-PCR amplification among the 24 isolate fluorescent Pseudomonads; PB-Number of polymorphic bands; MB-Number of monomorphic bands; PIC- Polymorphism Information Content.

S.No	Primer code	The primer nucleotide sequence (5' to 3')	Total alleles	PB	MB	% PB	% MB	PIC
1	OPA-01	CAGGCCCTTC	9	9	0	100	0	0.34
2	OPA-11	CAATCGCCGT	8	8	0	100	0	0.31
3	OPA-17	GACCGCTTGT	9	7	2	77.78	22.22	0.12
4	OPD-01	ACCGCGAAGG	12	12	0	100	0	0.36
5	OPD-02	GGACCCAACC	11	11	0	100	0	0.27
6	OPD-03	GTCGCCGTCA	5	3	2	60	40	0.20
7	OPE-02	GGTGCGGGAA	5	3	2	60	40	0.05
8	OPE-04	GTGACATGCC	5	5	0	100	0	0.28
9	OPF-10	GGAAGCTTGG	12	12	0	100	0	0.25
10	OPF-11	TTGGTACCCC	5	5	0	100	0	0.36
11	OPF-14	TGCTGCAGGT	5	5	0	100	0	0.24
12	OPC-12	TGTCATCCCC	1	0	1	0	100	0

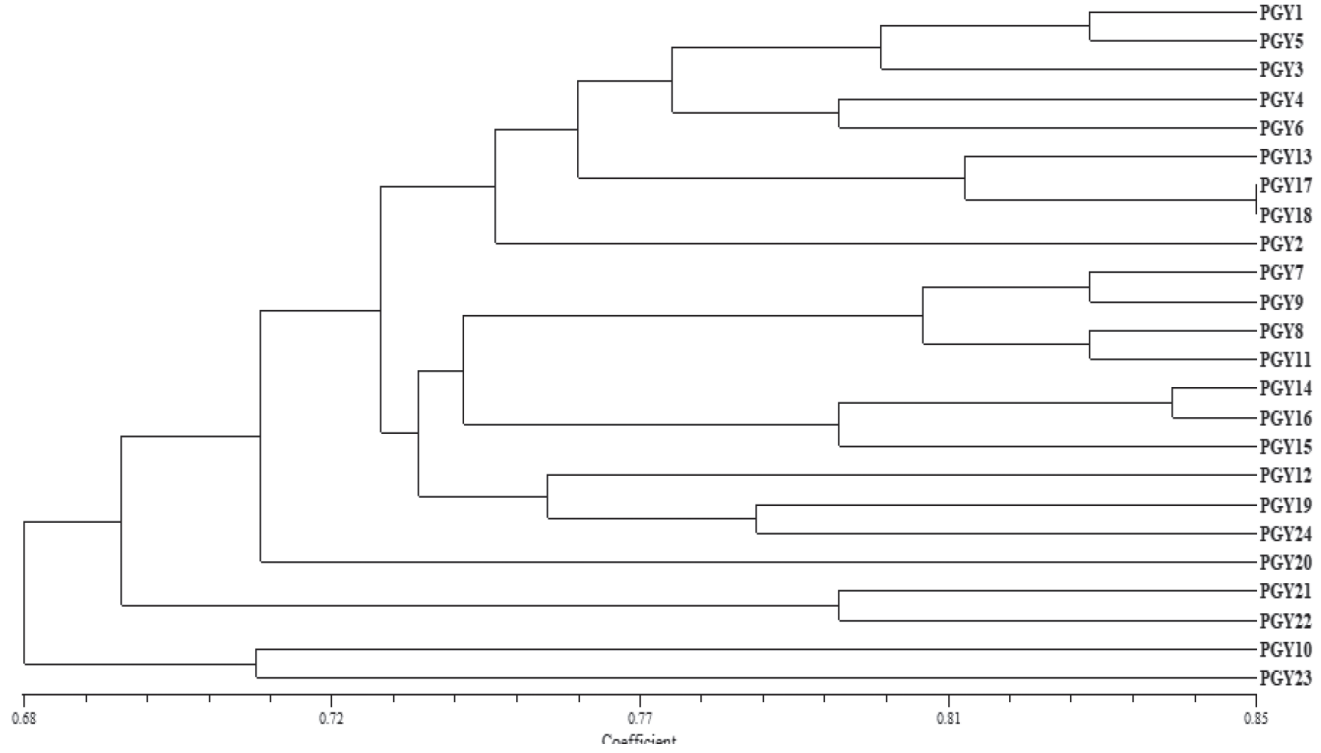

Figure 2: Dendrogram derived from unweighted pair group arithmetic mean (UPGMA) program using NTSYS pc 2.02j software showing the relationship among 24 fluorescent Pseudomonads bacterial isolates.

4. Discussion

In the present study, RAPD technique was successfully utilized for rapid characterization of 24 fluorescent *Pseudomonas* isolates of the Rayalaseema region of the State of Andhra Pradesh, India. The 24 isolates of fluorescent Pseudomonads genetic variability were categorized into different clusters and sub-clusters. Among the different molecular markers, Random Amplified Polymorphic DNA (RAPD) technique issuitableand is easy to execute as it does not have the need for any DNA sequence information of any species (Weder, 2002). Due to its technical simplicity, the use of RAPD as molecular markers for taxonomic and systematic analyses of plants and bacteria has increased. In-spite of the difficulty of reproducibility of RAPD molecular markers until in recent times, they are the well-known markers in terms of price and are widely used to study all living species genetic diversity. The RAPD molecular markers sequence information homology is restricted to the 10 bases of each amplification product. RAPD has been used for the estimation of genetic diversity in various endangered plant species and bacteria (Wang *et al*., 2005; Lu *et al*., 2006; Rayar *et al*., 2015).

A total of 12 different operon series primers were used to assess the 24 isolates and a total of 87 alleles were scored, of which 80 (91.95%) were polymorphic (Table 1). Out of 12 oligonucleotide RAPD primers, 8 primers produced 100% polymorphic bands, while three primers gave both mono and polymorphic alleles. These banding patterns revealed diverse raging of amplification patterns and a significant level of polymorphism. The total number of alleles noted for all markers were ranged from one to twelve and the maximum was noted by OPD-1 and OPF-10 primers (Table 1).Rameshkumar *et al*. (2011) RAPD data of Pseudomonad isolates isolated from rhizosphere of Sugarcane and its banding pattern revealed amplification product size ranged from 100 to 2,500 bp with three pgs RAPD primers and the dendrogram analysis differentiate the majority of isolates and they were grouped in different clusters. Asadhi *et al*. (2013) studied the genetic variability of Fluorescent Pseudomonads using RAPD markers and identified 8 to 17 numbers of amplified bands and the product sizes ranging from 100 to 3000 bp. The studied eight RAPD primers produced a total number of 99 amplified products with 100% polymorphism (Asadhi *et al*., 2013). The similarity coefficient of the sugarcane germplasm was ranged from 0.43 to 0.91 when thirty-five RAPD markers were used for genetic diversity assessment (Patel *et al*., 2018). Rayar *et al*. (2015) analyzed 17 isolates and reported 63.85% polymorphic bands with a genetic similarity ranged from 0.11 to 0.73. Singh (2015) studied and determined the genetic variability of plant growth-promoting rhizobacterial (PGPR) fluorescent Pseudomonads associated with chickpea (*Cicer arietinum* L.) rhizosphere using RAPD markers and concluded the existence of a low level of genetic variability in the species at 50% similarity level.

The dendrogram output categorized the fluorescent Pseudomonads isolated from different locations of the Rayalaseema region into two major clusters and two minor clusters. The results demonstrated thepresence of a degree of genetic variation among them. The genetic distance similarity matrix values for the 24 isolates ranged from 0.575 to 0.851. The highest similarity coefficient valuesof 0.851 were found between PGY17 and PGY18 and the lowest value of 0.575 was reported between PGY10 and PGY20. It is reported that the genetic diversity analysis of *Pseudomonas fluorescence* isolates collected from rice (*Oryza sativa*) rhizosphere from Southern India had an amplification range from 100 bp to 2 Kb with a similarity of approximately 25% to 95%. The diversity assessment of PGPR fluorescent Pseudomonads strains isolated from turmeric rhizosphere from Tamil Nadu, India, concluded that the RAPD amplified products varied from 100 to 2500 bp with a total of 92 alleles and a total of seven primers produced 100% polymorphic bands out of the 15 RAPD primers (Prabhukarthikeyan *et al*., 2019). Out of the 94 total alleles observed, 82 alleles were polymorphic and higher PIC was calculated for OPA01 followed by OPF10 (Prabhukarthikeyan *et al*., 2019). The dendrogram was constructed using a total of six primers which produced 296 polymorphic bands and the eightisolates were classified into two major classes and these *P. fluorescen*ce isolates were collected from the root zone of citrus (Koche *et al*., 2020).

Extensive investigation on fluorescent Pseudomonads isolates genetic diversity is essential to evaluate the intra-species diversity analysis. These diversity studies at the intra-species level will be useful in the conservation and development strategies. Based on the molecular genetic studies and similarity index, all isolates collected from Anantapur district were classified in sub-cluster-I with a similarity coefficient value of more than 0.75, and three isolates collected from Kadapa district were categorized in the sub-cluster-II of major cluster-I. The cluster-II mainly consists of three sub-clusters and most of the samples were collected from the Kurnool, YSR Kadapa and Chittoor districts of the Rayalaseema region. The samples collected from Anantapur and Chittoor districts were placed distantly from each other. The isolates collected from Anantapur and few isolates of Kadapa may be analogousto each other and the few isolates of Kurnool and Kadapa may be analogous to each other. It is also apparent that three of the isolates

collected from the Chittoor district were separated from most of the isolates. Genetic diversity assessment of strains from the different forest areas of the Western Ghats of Uttara Kannada District, Karnataka, revealed 37 strains as Pseudomonas fluorescence, 13 isolates as *Pseudomonas aeruginosa*, and two isolates as *Pseudomonas aureofaciens* (Megha *et al*., 2007).

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Antibacterial activity of saffron stigma and leaf extracts against human pathogenic bacteria

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ABSTRACT

Pathogenic microbes are detrimental to human health. On the other hand, several drugs and antibiotics have already been losing their effectiveness in killing the pathogens. Therefore, it is imperative to explore new drugs using extracts of medicinal plants with improved antimicrobial activity and relatively less side effects. Thus, antibacterial activity of hydroethanolic extracts of saffron leaves and stigma have been studied against 06 Gram-negative and 03 Gram-positive human pathogenic bacterial strains. Herein, antibacterial activity of saffron stigma and leaf extracts were observed in different concentrations (5, 10, and 15 mg/ml) against 09 pathogenic bacterial strains. Results suggested that, both the extracts significantly ($p < 0.05$) retarded the growth of bacterial strains. Further, stigma extract was more effective against *S. flexneri*, *L. monocytogenes*, *S. aureus* and *K. pneumoniae*, where as leaf extract was more effective against the growth of *S. flexneri*, *S. aureus* and *S. pneumoniae*. However, higher concentrations of both the extracts inhibit growth of *P. aeruginosa*. Hence, further research is highly essential relating to the bioactive compound of extracts and its mode of action in inhibiting growth of pathogenic bacteria for drug development.

1. Introduction

Infectious diseases caused by pathogenic microbes such as bacteria, fungi, viruses and parasites are associated with health risks. In the recent decade, the severity of diseases and the pathogenicity of microbes are considered to be a major concern for medical sciences. According to a recent report, most infectious diseases account for nearly 4.3 million deaths in 2016 (World Health Organization, 2019). Now the use of antibiotics has been increased for treatment of various diseases. However, it may kill the infectious and residual microbes inside the body that are not fatal but helpful (Langdon *et al*., 2016). This can also cause serious allergies, vomiting, headache and swelling of the face. Furthermore, some antibiotics and life-saving drugs lose their effectiveness against many diseases as infectious bacteria and other microbes develop resistance to them (Zaman *et al*., 2017; Fair and Tor, 2014). Antimicrobial resistance (AMR) is considered to be a serious concern to public health, as it deals with microbial resistance to antibiotics or any effective treatment previously generated for those microbes. Globally, around 700 thousand deaths per year are due to antimicrobial resistance (Capozzi *et al*., 2019).

The side effects and resistance of microbes to antibiotics led to increased interest in new approaches of using medicinal plants for drug development (Muzaffar *et al*., 2016). Plant metabolites have significant antimicrobial properties and their anti-mutagenic properties prevent mutation in bacteria thereby reducing bacterial antibiotic resistance (Gupta and Birdi, 2017). Nowadays a number of plants have been recognized for their medicinal value and are used as source of various chemical compounds that act against pathogenic microbes. Mostafa *et al*. (2018) reported the antibacterial activity of ethanolic extracts of *Punica granatum*, *Syzygium aromaticum*, *Zingiber officinales, Thymus vulgaris* and *Cuminum cyminum* against

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Staphylococcus aureus, *Bacillus cereus*, *Escherichia coli*, *Salmonela typhi* and *Pseudomonas aeruginosa*. Methanolic extracts of *Oxalis corniculata*, *Artemisia vulgaris*, *Cinnamomum tamala* and *Ageratina adenophora* showed variable antibacterial efficiencies against *Escherichia coli*, *Salmonela typhi*, MDR *Salmonela typhi*, *Klebsiella pneumoniae*, *Citrobacter koseri,* and *Staphylococcus aureus* (Manandhar *et al*., 2019). Gonelimali *et al*. (2018) investigated the antibacterial activity of ethanolic and aqueous extract of *Hibiscus sabdariffa*, *Rosmarinus officinalis*, *Syzygium aromaticum* and *Thymus vulgaris* against some food poisoning bacteria viz., *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa*.

The medicinal saffron plant (*Crocus sativus* L.) is a monocot, sterile, triploid plant, that belongs to the family, Iridaceae and isvegetatively propagated by corms. Saffron stigmas contain more than 150 potential chemical compounds such as carotenoids, precursor compounds of many apocarotenoids such as crocin, picrocrocin, and safranal (Shahi *et al*., 2016). However, recent reports suggested that other parts of the plant (petals, leaves and corms) also contains a number of chemical compounds (Maqbool *et al*., 2022). The medicinal values of many chemical compounds such as anti-inflammatory, anti-depressantant neuro protective, antioxidant & memory enhancing effect, cytotoxic and anti-cancer effect, and antibacterial effect has already been discovered by many researchers (Hosseinzadeh and Younes., 2002; Feizzadeh *et al*., 2008; Zhang *et al*., 2013; Nam *et al*., 2010; Potnuri *et al*., 2018; Papandreou *et al*., 2011; Arzi *et al*., 2018; Samarghandian *et al*., 2013; Aung *et al*., 2007; Nair *et al*., 1995; Pintado *et al*., 2011). Recent studies reported that saffron leaves contain high phenolic compounds, organic acids, naringenin, quercetin and apigenin which has antibacterial activity against different pathogenic bacteria (Jadouali *et al*., 2018; Mykhailenko *et al*., 2021). On account of that, an attempt has been made to evaluate the antibacterial activity of ethanolic extracts of saffron stigma and leaves against nine human pathogenic bacterial strains.

2. Materials and methods

2.1. Collection of plant materials

Saffron corms were collected in the month of June 2022 from the Saffron research station, in Kashmir. The disease-free corms were subsequently soaked in 0.2% of Bavistin fungicide solution and dried for 2-3 h. Then the corms were incubated at 25^{0}C under dark conditions at 85 ± 2% humidity for 3 months (Eftekhari *et al*., 2023). Around 2.5-3 cm larger corms were planted in pots. The pots were kept inside the greenhouse with temperature 20^{0}C in day and 17^{0}C at night and watered every two days. The flowers appeared in the last week of August after the shoot heighted about 1-3 cm. Then the flowers were harvested and the stigma was plucked up followed by shade dried at room condition. The vegetative growth of plants was observed after flowering & the leaves were collected after 45 days and subjected to shade drying followed by oven-drying at 50-60^{0}C for 72 hrs.

2.2. Preparation of ethanol extract of leaves and stigma

Properly dried stigma and leaves were used for the preparation of ethanol extract. Each sample was macerated separately with mixtures of aqueous and ethanol (8:2) at a concentration of 1 gm / 20ml. The ground materials were further centrifuged at 3000 rpm for 15 min, and the supernatants were collected. The process was repeated two times & both the extracts were evaporated and dried under reduced pressure at 40^{0}C using a rotary vacuum evaporator (Fig 1). Crude extracts were freeze-dried and stored at -20^{0}C until further use. The yield percentage (%) of both extracts were calculated by the formula (Mostafa *et al*., 2018).

Yield percentage of extract (%) = Weight of extract after evaporation of the solvent (W_1) X 100 / Dry weight of plant raw sample (W_0)

2.3. Bacterial strain used in this study

The antibacterial activity was investigated against nine bacterial species. Among them, six strains were gram negative (*Shigella flexneri* MTCC 1457, *Escherichia coli* ATCC 25,922, *Salmonella typhimurium* MTCC 3224, *Klebsiella pneumoniae* MTCC 3384, *Pseudomonas aeruginosa* Chl01, *Escherichia coli* K12SMTCC 728) and three were gram positive bacteria (*Listeria monocytogenes* MTCC 1143, *Streptococcus pneumoniae* MTCC 1936, *Staphylococcus aureus* ATCC 25,923) collected from Imgenex India, Bhubaneswar and Environmental microbiology laboratory, Ravenshaw University, Cuttack, Odisha. These bacterial strains cause several diseases in human shown in (Table 1).

2.4. Preparation of inoculums

Bacterial strains were cultured in nutrient broth (LB medium) overnight in an incubator shaker at 37^{0}C. According to 0.5 McFarland standards, each strain culture was adjusted to 108 CFU/ml.

2.5. Antibacterial activity

The in vitro antibacterial activity was carried out by the agar well diffusion method in LB agar plates. The nutrient agar plates were inoculated with 100µl of each microbial suspension and spread uniformly by using a sterile spreader.

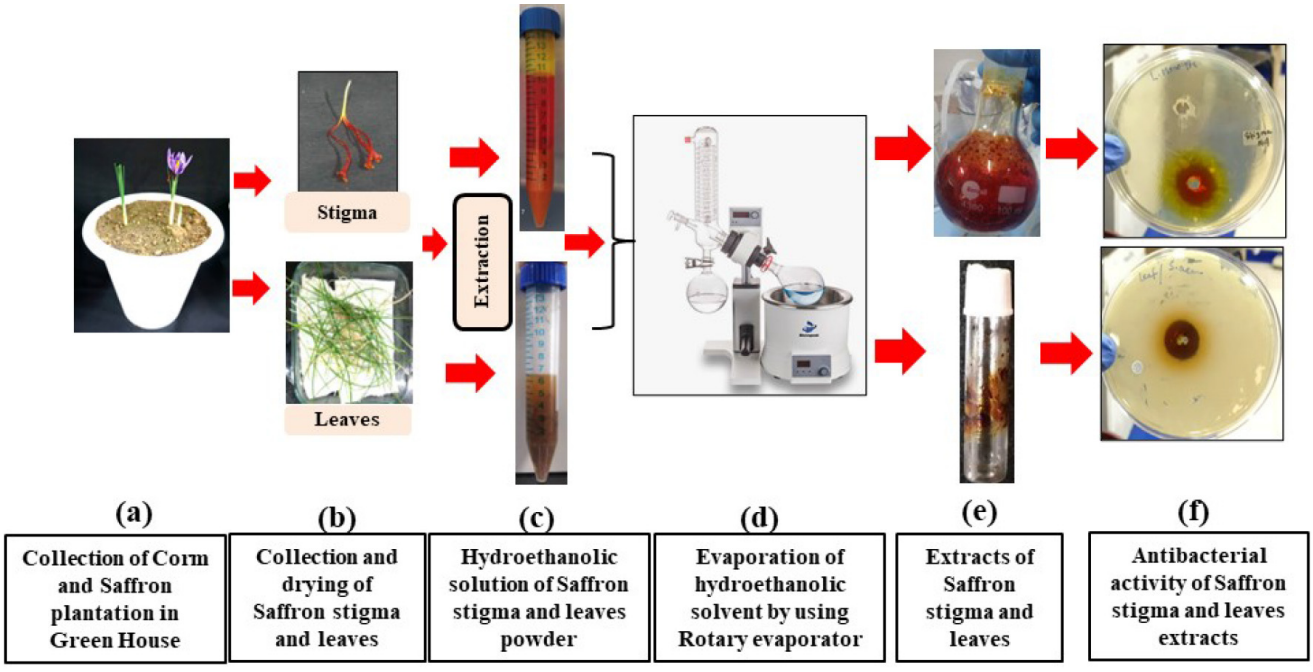

Figure 1: Various steps of hydroethanolic extracts preparation of saffron stigma and leaves to study the antibacterial activity.

Table 1:

List of bacterial disease in human and their causal agent

Bacterial strain name	Gram +/-	Causing disease in human	Reference
Shigella flexineri	Gram (-)	Cause diarrhoea in human	Zaidi and Estrada-Gracia, 2014
Escherichia coli	Gram (-)	Cause bloody diarrhea, urinary tract infections, meningitis etc.	Clements *et al.,* 2012
Listeria monocytogenes	Gram (+)	Causes listeriosis	Jemmi and Stephan, 2006
Staphylococcus aureus	Gram (+)	Cause skin infections	Kobayashi *et al.,* 2015
Streptococcus pneumoniae	Gram (+)	Cause of pneumonia	Weiser *et al.,* 2018
Klebsiella pneumoniae	Gram (-)	Cause pneumonia, urinary tract infections, blood stream infections, sepsis	Bengoechea and Sa Pessoa, 2019
Salmonella typhi	Gram (-)	Causative agent of typhoid fever	Kidgell *et al.,* 2002
Pseudomonas aeruginosa	Gram (-)	Cause nosocomial infections	Fazeli *et al.,* 2012

Then 6 mm diameter of well was prepared using a sterile tip. Stock solutions were prepared by dissolving each extract in DMSO. Then three different concentrations (5, 10, 15 mg/ml) of stigma and leaf extracts were added to the well and incubated at 37°C for 12-18 hrs. Standard antibiotics such as ampicillin and gentamycin were used as positive control while DMSO as negative control. The positive antibacterial activity was recorded on the basis of growth inhibition.

2.6. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of antibacterial substance that inhibits the visible growth of microorganisms tested (Balouiri *et al.,* 2016). In this study, MICs for leaf and stigma extract were evaluated by the macro broth dilution method (Motamedi *et al.,* 2010). In macro dilution method, concentrations of leaf and stigma extracts ranged from 0 to 50 mg/ml were added to tubes in reference to the concentration of extract responsible for the production of inhibition zones in the antibacterial assay. Each tube containing 1 ml of nutrient medium was inoculated with standardized bacterial suspension adjusted to the 0.5 McFarland scale and incubated at 37°C for 12-18 hrs.

2.7. Statistical analysis

Here, all the experiments were carried out with three replicates, and the data were represented as mean ± standard error of the mean. Results were analyzed through a two-way analysis of variance and the mean was compared by performing Tukey's multiple comparisons test (GraphPad Prism 8.0.1.244). The significant difference was considered at $p< 0.05$.

3. Result and Discussion

3.1. Yield of plant extract

The hydroethanolic extracts of stigma and leaves were harvested from saffron plants (Fig 1). 10 g of leaf and stigma extract of saffron plant sample yielded 4.53 g and 3.12 g of residue from the stigma and leaves extract respectively. So, the yield of stigma extract was comparatively higher than leaf extract.

3.2. Antibacterial activity

Saffron leaves and stigma extract residues were evaluated for antibacterial activity against nine human pathogenic bacterial strains. The antibacterial activity was determined by using agar well diffusion method and effect of both the extracts were observed at concentrations of 5, 10, and 15 mg/ml for all the bacterial strains. The results obtained from this assay revealed that both the extract significantly retarded the growth of bacterial strains (Table 2).

Stigma extract was significantly more effective ($p < 0.05$) against the growth of *S. flexneri, E. coli, L. monocytogenes, S. aureus, S. pneumoniae* and *K. pneumonia* (Fig 2 and 3). The increasing concentration of extracts correspondingly increased the inhibition of bacterial growth. Motamedi *et al.* (2010) also evaluated the antibacterial activity of ethanolic extract of *C. sativus stigma* at a concentration of 50, 100, 200, and 400 mg/ml against *Brucella melitensis*. Effective antibacterial activity of ethanolic extract of stigma (7.5 mg/ml) was observed by against *E. coli* and *S. aureus*, while no antibacterial activity was observed against *K. pneumoniae* (Mir *et al.,* 2011). This may be because of the use of saffron from a different locality. The climatic conditions might involve differences in the effectiveness of saffron extracts against pathogenic bacterial species.

Stigma of saffron is the source of a wide variety of chemical compounds and among them, crocin, picrocrocin, and safranal contribute the important active constituents (Carradori *et al.,* 2016; Shahi *et al.,* 2016; Gohari *et al.,* 2013; Srivastava *et al.,* 2010). Safranal and crocins are volatile and

Table 2:

Antibacterial activity of saffron leaf and stigma hydroethanolic extract against pathogenic bacteria; ND: Inhibion zone Not Determined, SF: *Shigella Flexneri*, EC: *Escherichia coli,* ECK *K12S*: *Escherichia coli K12S,* LM: *Listeria monocytogenes,* SA: *Staphylococcus aureus*, SP: *Streptococcus pneumoniae*, KP: *Klebsiella pneumoniae,* ST: *Salmonella typhi,* PA: *Pseudomonas aeruginosa*

Bacterial strain	Antibacterial activity (mm)					
	Stigma extract	Leaf extract	Stigma extract	Leaf extract	Stigma extract	Leaf extract
	5 mg/ml		10 mg/ml		15 mg/ml	
SF	4.1±0.231^{ab}	6.2±0.265	6.833±0.203^{a}	10.4±0.321^{b}	10.467±0.481	15.333±0.273
EC	3.067±0.145^{a}	ND	5.267±0.176	1.2±0.153	8.467±0.433	3.4±0.1
ECK*K12S*	ND	1.667±0.285	2.067±0.145	4.867±0.176	3.3±0.265^{a}	8.167±0.176
LM	6.267±0.233^{cd}	ND	10.067±0.437^{cd}	ND	15.233±0.260^{cd}	ND
SA	4.367±0.285^{b}	4.067±0.318^{a}	8.033±0.353^{ab}	9.133±0.463^{a}	13.567±0.348^{b}	13.733±0.26^{a}
SP	6±0.404^{c}	4.267±0.233^{a}	9.2±0.346^{bc}	9.7±0.265^{ab}	14.9±0.265^{cd}	14.133±0.26^{a}
KP	6.333±0.41^{cd}	ND	9.433±0.291^{cd}	ND	14.233±0.318^{bc}	ND
ST	1.367±0.176	1.167±0.167	3.433±0.273^{A}	3.1±0.173	4.233±0.176^{aA}	5.867±0.47
PA	ND	ND	ND	ND	ND	ND

Values in the table are means ± Standard deviation of three replicates (n = 3)

Values with different lowercase letter (a-d) in the same columns differ significantly ($p < 0.05$)

Values with same uppercase letter (A) in the same row not significantly differ from each other

water-soluble compounds, thus can easily reach pathogenic bacteria and inhibit their growth (Pintado *et al.,* 2011). The maximum inhibition of bacterial growth by stigma extract was obtained with *L. monocytogenes* (6.267±0.233 mm) and *K. pneumoniae* (6.333±0.41 mm) at a concentration of at 5 mg/ml. Even at higher concentrations of stigma extract (10 and 15 mg/ml), insignificant level of inhibition (3.433±0.273 and 4.233±0.176 respectively) was seen with *S. typhimurium*. *Escherichia coli* K12S showed resistance against saffron stigma extracts at a concentration of 5 mg/ml, while its growth inhibition was observed at higher concentrations of 10 and 15 mg/ml.

The selection of an appropriate solvent also plays a crucial role in extracting compounds of interest from the sample (Truong *et al.,* 2019). Shahidi *et al.,* (2008) reported that polar solvents are best forextraction of effective active constituents from saffron. Many studies reported that, polar solvents such as methanol, acetone, ethyl acetate, ethanol, distilled water, etc. have been used for the extraction. Ethanol has higher polarity than methanol, acetone and ethyl acetate. Methanol also has bleaching properties as it reduces the colouring content of the extract (Sani and Mohseni, 2014). It was also reported that a mixture of aqueous and ethanol is considered as most effective in extracting crocin, picrocrocin, and safranal from saffron (Gazerani *et al.,* 2013). Herein, ethanol was taken as a solvent for extraction of both the saffron stigma and leaf sample.

The leaf extract wassignificantly more effective against the growth of *S. flexneri, S. aureus* and *S. pneumonia* ($p < 0.05$) in comparison to other bacterial growth (Fig 4 and 5). The maximum inhibitory zone at 5 mg/ml of leaf extract was found in S. flexneri (6.2±0.265 mm), followed by *S. aureus* and *S. pneumoniae* formed 4.067±0.318 and 4.267±0.233 mm of inhibitory zone respectively. The minimum inhibition zone was found in *S. typhimurium* (1.167±0.167) and *Escherichia coli* K12S (1.667±0.285 mm). The antibacterial activity of ethanolic extract of leaves at a concentration of 100 mg/ml against *S. aureus* was reported by Okmen *et al.* (2016). However, Vahidi *et al.,* (2002) didn't observe any antibacterial activity against *S. aureus* and *E. coli* in response to ethanolic extract of leaves at 100 mg/ml concentration. Increased concentrations of methanol extracts of leaves showed effective antibacterial activity against *Listeria* spp. (Jadouali *et al.,* 2017). Leaves of Saffron constitute a source of bioactive compounds with different physiological activities and possible applications. Crocus leaves have a higher percentage of protein, lipids, total carbohydrates and total phenolic content than those of the petals. The leaf extract also exhibited higher antioxidant capacity (Jadouali *et al.,* 2017). Mykhailenko *et al.,* (2021) recently reported 16 compounds from saffron leaf extracts among which two major active compounds, mangiferinand isoorientin were found. They also identified some unique compounds such as tectoridin, iristectorigenin B, nigricin, and irigenin in leaves of saffron.

Some bacterial strains such as *L. monocytogenes, K. pneumoniae* and *P. aeruginosa* showed resistance to leaf extract at the supplemented concentrations. However, one bacterial strain *P. aeruginosa* exhibited resistance against stigma extract. Earlier reports suggested that *P. aeruginosa* is resistant to most of the available antibiotics (Tummler, 2019). Mir *et al.* (2011) also observed no antibacterial activity of the ethanolic extract of stigma against *P. aeruginosa*. Whereas, significant antibacterial activity was reported against *P. aeruginosa* at 1000 µg/disk concentrations of petroleum ether and methanolic extracts of stigma respectively (Muzaffer *et al.,* 2016). Mykhailenko *et al.* (2021) recently reported the effect of ethanolic and water extracts of Saffron leaves from Ukraine showed significant antibacterial activity against *Bacillus subtilis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa* and *Candida albicans.* Jadouali *et al.* (2018) reported that methanolic extract of saffron leaves from Morocco plants did not show antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa.* As a result, it may be inferred that saffron plants from different regions may have varying levels of efficiency in reducing bacterial growth.

Significant antibacterial activity was observed by ampicillin and gentamycin against all bacterial strains whereas; DMSO did not interfere with the growth of bacterial strains. As the concentration of extracts increased, antibacterial activity also increased significantly against bacterial strain ($p < 0.05$). Further, similar observations were also reported (Soureshjan and Heidari, 2014; Muzaffar *et al.,* 2016).

3.3. Minimum inhibitory concentration

Minimum inhibitory concentrations (MIC) of plant extract means a minimum concentration of extract to inhibit the growth of bacterial strain (Balouiri*et al*., 2016). MIC of both the extract was measured for all the bacterial strains shown in (Table 3). Low concentrations of stigma extract were determined as MIC against *S. flexneri*, *K. pneumoniae, S. pneumoniae* and *L. monocytogenes* (3.5 mg/ml, 4.5±0.289 mg/ml, 5.333±0.441mg/ml, and 5.5±0.289 mg/ml respectively). The inhibitory effect of leaf extract is most effective against *S. flexneri*, *S. aureus,* and *S. pneumoniae*, so the MICs of the extract are less for these strains (4.5±0.289 mg/ml, 5±0.289 mg/ml, and 5.5±0.289 mg/ml respectively). The MIC of stigma

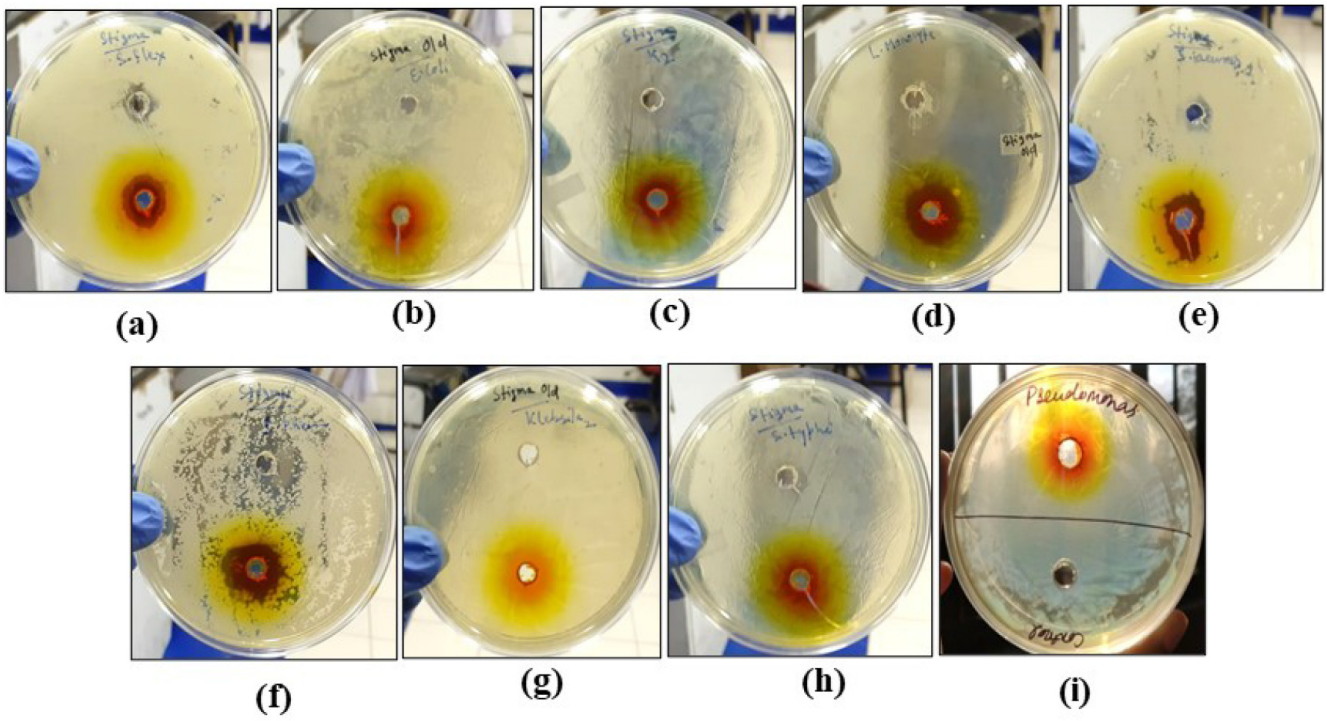

Figure 2: Antibacterial activities of ethanolic extract of stigma using agar well diffusion method showing inhibition zone; (a) *Shigella Flexneri,* (b) *Escherichia coli,* (c) *Escherichia coli K12S,* (d) *Listeria monocytogenes,* (e) *Staphylococcus aureus,* (f) *Streptococcus pneumoniae*, (g) *Klebsiella pneumoniae, (h) Salmonella typhi,* (i) *Pseudomonas aeruginosa*.

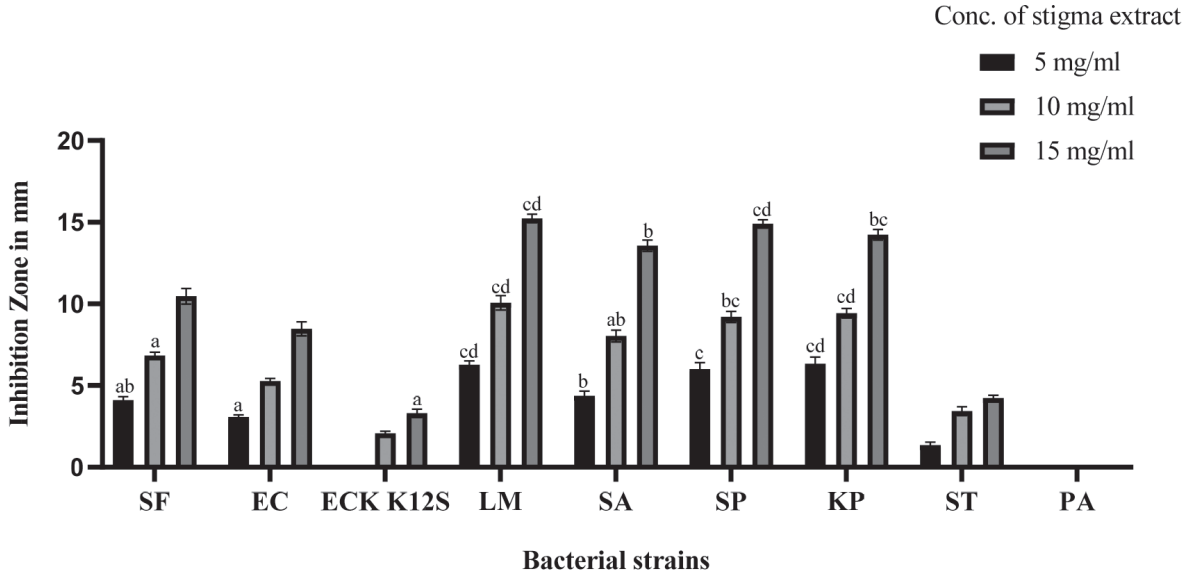

Figure 3: Antibacterial activity by stigma extract; SF: *Shigella flexneri*, EC: *Escherichia coli,* ECK K12S: *Escherichia coli* K12S, LM: *Listeria monocytogenes,* SA: *Staphylococcus aureus*, SP: *Streptococcus pneumoniae*, KP: *Klebsiella pneumoniae,* ST: *Salmonella typhi,* PA: *Pseudomonas aeruginosa*. Values with different lowercase letters (a-d) in the same concentration of extract differ significantly ($p < 0.05$).

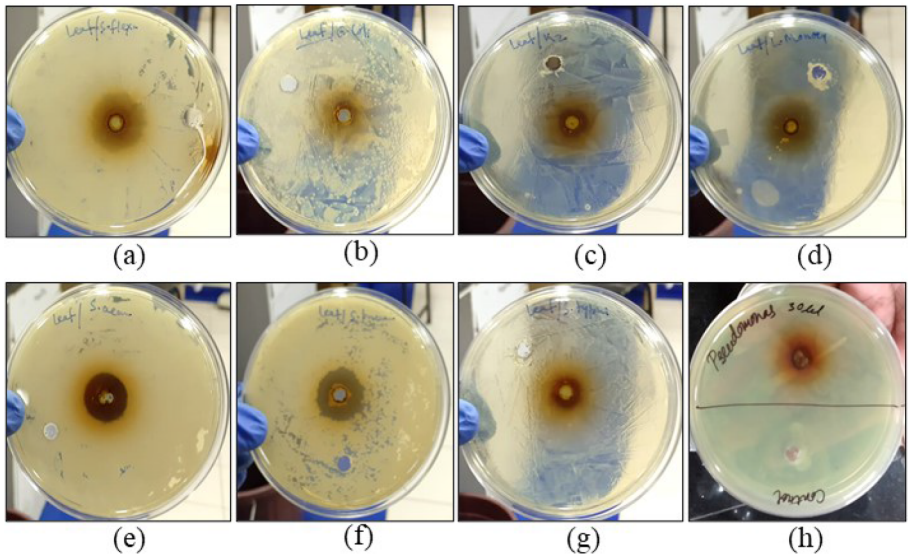

Figure 4: Antibacterial activities of ethanolic extract of leaves using agar well diffusion method showing inhibition zone; (a) *Shigella flexneri,* (b) *Escherichia coli,* (c) *Escherichia coli* K12S, (d) *Listeria monocytogenes,* (e) *Staphylococcus aureus,* (f) *Streptococcus pneumoniae*, (g) *Salmonella typhi, (h) Pseudomonas aeruginosa.*

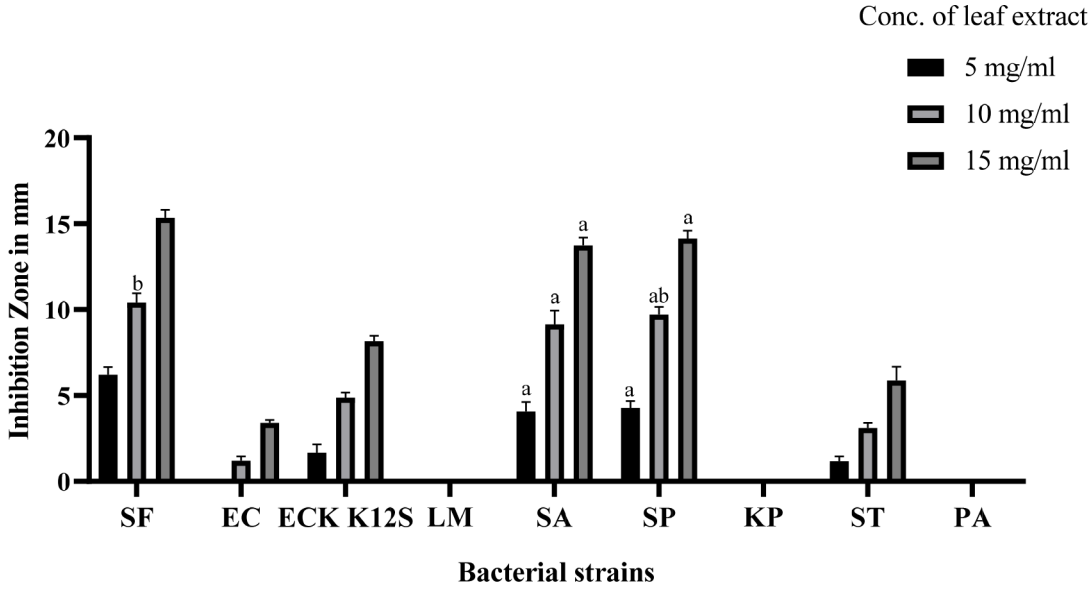

Figure 5: Antibacterial activity of leaf extract; SF: *Shigella flexneri*, EC: *Escherichia coli,* ECK K12S: *Escherichia coli* K12S, LM: *Listeria monocytogenes,* SA: *Staphylococcus aureus*, SP: *Streptococcus pneumoniae*, KP: *Klebsiella pneumoniae,* ST: *Salmonella typhi,* PA: *Pseudomonas aeruginosa*. Values with different lowercase letters (a-d) in the same concentration of extract differ significantly ($p < 0.05$).

extract was found at high concentrations for *P. aeruginosa* (35.500±0.289 mg/ml) whereas, the MIC of leaf extract was observed at high concentrations for *K.pneumoniae, P. aeruginosa*, and *L. monocytogenes* (34.667±0.333 mg/ml, 32±0.289 mg/ml, and 27.5±0.289 mg/ml respectively). Higher concentrations of stigma and leaf extracts were effective in reducing the growth of *P. aeruginosa*. A study reported the MIC of methanolic extract of stigma was 200 ± 0.45, 500 ± 0.45, 300 ± 0.25, and 400 ± 0.15 µg/ ml for *S. aureus*, *E. coli*, *P. aeruginosa* and *S. flexneri* respectively (Parray *et al*., 2015).

Table 3:

Minimum inhibitory concentration of saffron leaf and stigma hydroethanolic extract against pathogenic bacteria; ND: Inhibion zone Not Determined, SF: *Shigella Flexneri*, EC: *Escherichia coli,* ECK*K12S*: *Escherichia coli K12S,* LM: *Listeria monocytogenes,* SA: *Staphylococcus aureus*, SP: *Streptococcus pneumoniae*, KP: *Klebsiella pneumoniae,* ST: *Salmonella typhi,* PA: *Pseudomonas aeruginosa*

Bacterial strain	Minimum Inhibitory Concentration (MIC) (mg/ml)	
	Plant sample ethanolic extract	
	Stigma extract	Leaf extract
SF	3.5±0.00^{a}	4.5±0.289^{a}
EC	6.00±0.289^{bcd}	11.5±0.289
ECK*K12S*	12.667±0.167^{e}	9.5±0.289^{c}
LM	5.5±0.289^{bc}	27.5±0.289
SA	6.333±0.441^{bcd}	5±0.289^{ab}
SP	5.333±0.441^{b}	5.5±0.289^{ab}
KP	4.5±0.289^{a}	34.667±0.333
ST	12±0.289^{e}	8.5±0.289^{c}
PA	35.500±0.289	32±0.289

Values in the table are means ± Standard error of mean of three replicates (n = 3)

Values with different lowercase letter (a-e) in the same columns differ significantly ($p < 0.05$)

4. Conclusion

This study concluded that hydroethanol extract from both saffron stigma and leaf samples have significant anti-bacterial activity against several human pathogenic bacteria. A progressive increase in the concentration of both extracts resulted in a larger inhibition zone. However, the stigma extracts showed effective antibacterial activity by inhibiting the growth of a maximum number of pathogenic bacteria in comparison to the leaf extract. Hydroethanol was also an adequate solvent in extracting important bioactive compounds from saffron samples. Extracts of these two samples (leaf and stigma) of saffron plant can be analysed in future for further assessment of different bioactive compounds that may have applications in various pharmacological industries.

Disclosure statement

All the authors declare that there is no conflict of interest.

Authors contributions

Khirod Kumar Sahoo designed, conceptualized, supervised, and participated in writing the manuscript. Namita Muduli and Purusottam Ojha performed the experiments, analyzed and interpreted the data, and wrote the manuscript. All have given their consent for approval of the final publication of the manuscript.

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Polyhydroxyalkanoates production by sugarcane rhizospheric soil bacterial isolate

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ABSTRACT

PHAs are the biopolymers synthesized by a wide array of bacteria as carbon and energy storage granules. However, its synthesis by sugarcane rhizospheric soil bacteria has gained the utmost attention due to its utilization of a broad spectrum of synthetic or inexpensive substrates. Herein, 20 aerobic bacteria were isolated from the rhizospheric soil of sugarcane using standard bacteriological techniques. Among them, 03 Gram positive bacterial isolates (B1, B2 & B3) were able to accumulate PHAs granule in their cytosol as confirmed from Sudan black B staining. Based on the intensity of staining, bacterial isolate B1 was selected for further study. Under solid-state fermentation (SSF), bacterial isolate B1 was found to produce 1.2 g/l of PHAs using sucrose as carbon source. The bacterial isolate B1 was identified as *Bacillus* sp. B1 by morpho-physiological characterization. Further, optimization of process parameters, characterization of PHAs and species level identification of the bacterial isolate is highly essential in this regard.

1. Introduction

Synthetic plastic production has become inevitable in the world and are used in different sectors of operations. It was estimated that about 187 million tonnes (Mt) of petroleum-based plastic are generated per year globally (Chavan *et al*., 2021). However, their excessive use because of their mechanical integrity, excellent durability, cost-effectiveness and easy production, it is accumulating in the environment leading to environmental pollution (Narayanan *et al*., 2020). Hence, these petroleum-based plastics need to be replaced by bioplastics. Polyhydroxyalkanoates (PHAs), act as a suitable alternative to conventional plastics produced by various microbes including bacteria, archaea, cyanobacteria and plants as energy storage granules (Maity *et al*., 2020). Depending on the presence of monomer, these are categorized into three categories viz., short chain length (*scl*), medium chain length (*mcl*) and long chain length (*lcl*) PHAs. The molecular weight of PHAs varies between 200,000 - 2000,000 Dalton depending on desired bacterial strain, fermentation conditions and substrate used in the bioprocess technology (Mohapatra *et al*., 2020). Among all, polyhydroxybutyrate (PHB), poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), poly (3-hydroxybutyrate-co-4-hydroxybutyrate) (P3HB4HB) and poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) have been produced on a mass scale for commercial applications (Maity *et al*., 2017; Mohapatra *et al*., 2015). PHAs possess characteristics similar to synthetic plastics whereas, these are biodegradable and biocompatible under natural conditions to produce CO_2 and H_2O. Therefore, it is produced industrially and used in a broad spectrum of end products, ranging from packaging to medical applications (Amaro *et al*., 2019).

Several research on bioplastics suggest that due to genetic stability, easy cultivation and fast-growing ability, bacteria are the key organisms for the production of PHAs (Mohapatra *et al*., 2017). All at once, they can be biodegraded

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by living organisms by the process of decomposition leading to the formation of very small compounds by microbial activity (Aragosa *et al*., 2020). Microbes inhabiting different ecological niches such as estuaries, marine water, rhizospheric regions, groundwater and sewage accumulate PHAs granules due to stress experienced by them. These locations are often rich in organic contents to support actively involved microorganisms for PHA accumulation to meet the metabolic energy requirement. *Bacillus licheniformis, Bacillus cereus, Bacillus badius* are some of the reported Gram-positive PHAs producing bacteria isolated from rhizospheric soil regions of different plants like rubber, sugarcane etc (Mohapatra *et al*., 2015). The sugarcane rhizospheric region is known to be a hot spot of microbial activities since roots release several different organic compounds inform of exudates and mucilage which serve as adequate nutrient supply for microbes (Mohapatra *et al*., 2015). Therefore, the rhizospheric region is a highly favorable habitat for the proliferation, activity and metabolism of numerous microbes. Moreover, the predominance of amino acids and growth factors required for bacteria, are readily provided by the root exudates in the rhizospheric soil region. On the other hand, *Bacillus* species are the predominant soil-inhabiting bacteria thatcan grow by utilizing cheap raw material for their growth and development as well as accumulating PHAs (Dash *et al*., 2014). In light of the above, an attempt has been made to study PHA production by sugarcane rhizospheric soil bacterial isolate.

2. Materials and methods

2.1. Isolation of rhizospheric soil bacteria

Rhizospheric soil samples of sugarcane were collected from the agriculture field, Dhauli, Bhubaneswar, Odisha. Representative samples were collected using a sterile vial and then transported aseptically to the laboratory for bacteriological analysis. The collected samples were processed in the laboratory at the earliest to isolate rhizospheric soil bacteria and to study their morphology and other characteristic features required for their generic level identification. Ten-fold dilution followed by a spread plate method was performed to isolate desired bacteria. The required amount of sterile nutrient agar (NA) medium was prepared and poured into ten different sterile petriplates. Then, 0.1 ml of serially diluted sample from each dilution was spread to the respective petriplates and incubated at 37^0C for 24 hours. The colonies of distinct morphological characters were individually picked up, sub-cultured on NA medium and incubated at 37°C for 24 hours to obtain the pure culture. The resulting pure cultures were preserved in NA slants at 4^0C and also maintained in glycerol stock at -20°C for further characterization.

2.2. Screening of PHAs producer

Screening of the PHAs producing bacterial isolates was conducted by the Sudan black B staining method under bright field microscopic imaging (Pati *et al*., 2020). However, before the screening, the bacterial isolates were induced to accumulate PHAs granules by growing in nitrogen-limiting mineral salt medium (MSM) and incubated at 37°C for 48 hr. Then, the bacterial smear was flooded by 0.3% (w/v in 70% ethanol) of Sudan black B staining for 15 minutes followed by Gram's decolorizer for a few seconds and then counterstained with safranin (5% w/v in de-ionized water) for 10 seconds. The slides were then washed gently, dried and observed under light microscope (1000X, Leica DM5000B).

2.3. Generic level identification of PHAs producer

The PHAs producing bacterial isolates were subjected to Gram staining followed by biochemical characterization using the VITEK 2 Compact system (BioMerieux, France) (Yasin and Mayaly *et al.,* 2020). In this system, generic-level identification of bacterial isolates was conducted based on 63 different biochemical tests.

2.4. PHAs production

PHAs production via solid-state fermentation (SSF) was carried out using plate culture method. Briefly, 1L of MSM agar medium with pH 9.0 was prepared in 50 different petriplates and inoculum (24 hours fresh culture containing 1.5 x 10^8 cells/ml) was added following the lawn culture method and incubated at 37ºC for 72 hours. Then, PHA was recovered by following sonication and di-solvent extraction method. The harvested bacterial cell biomass was collected in 30 ml of acetone and the suspension was sonicated at 20 KHz/ power 100/ pulse 30s for 15 minutes. The cell biomass and filtrate (acetone extract) were collected, suspended in chloroform and evaporated at 70-80°C in a water bath to obtain PHAs film. Then, PHAs production (%) was estimated using the following formula (Pati *et al*., 2020; Mohapatra *et al*., 2016).

$$\text{PHAs production (\%)} = \frac{\text{Weight of PHAs}}{\text{Cell biomass (DCW)}} \times 100$$

3. Result and discussion

The rhizospheric soil region of sugarcane contains varied micro-flora due to the secretion of root exudates having adequate amount of carbon sources and inadequate amount of other nutrients. A wide array of bacteria is known to accumulate PHAs in the microhabitat where carbon concentration is higher and nitrogen content is lower

(Mohapatra *et al.,* 2015). These bacteria have been reported from various environments, but only a few from the rhizospheric soil region of sugarcane. On account of that, 20 different bacteria were isolated from the rhizospheric soil region of sugarcane (*Saccharum officinarum*). Among them, 03 bacterial isolates were found to accumulate PHAs in their cytosol as confirmed by Sudan black B staining (Fig. 1). Based on the intensity of staining, bacterial isolate B1 was selected for further study. Gram variability reactions revealed that, bacterial isolate B1 is Gram positive and rod in shape. Further, the bacterial isolate B1 was identified as *Bacillus sp.* B1 by morpho-physiological characterization.

Under SSF, the potent rhizospheric soil bacterial isolate *Bacillus sp.* B1 was found to produce 1.2 g/l of PHAs using synthetic carbon source. Our result coincides with recent data of PHAs production by different strains of *Bacillus* (Joseph *et al.,* 2021; Damle *et al.,* 2016; Mohapatra *et al.,* 2015; Dash *et al.,* 2014) through submerged fermentation process (SmF). However, reports are not available in the public domain for PHAs production via SSF using rhizospheric soil bacterial isolates. Researchers are still on their best path to isolate, identify and characterize potent PHAs producing bacterial isolates for better convenience in near future as these biopolymers represent a potential alternative to petrochemical-based plastics.

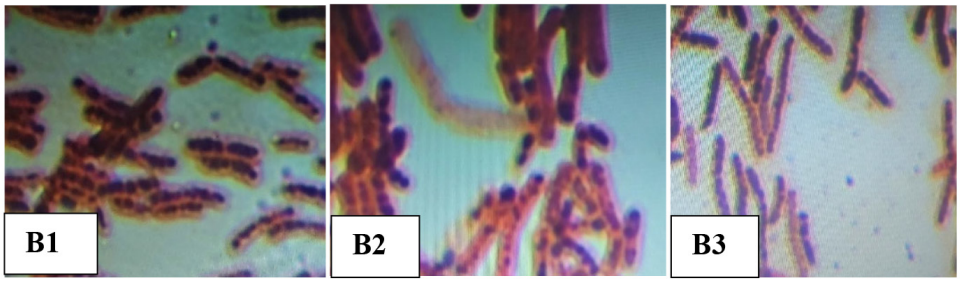

Fig 1: PHAs accumulating bacterial isolates B1,B2 and B3 under Sudan Black Bstaining

4. Conclusion

Under solid-state fermentation, the rhizospheric soil bacterial isolate *Bacillus sp.* B1 was found to produce 1.2 g/l of PHAs. However, optimization of process parameters, characterization of PHAs and species level identification of the bacterial isolate is highly essential in this regard.

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Growth stages modulate phytochemical content and antioxidant property in methanolic extract of *Vigna radiata* (L.) Wilczek leaves.

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ABSTRACT

Plant phytochemicals have extensive applications in the food, pharmaceutical and cosmetic industries. *Vigna radiata* (L.) Wilczek (mung bean) seeds and sprouts are consumed by humans as functional foods worldwide. In this study, we analyzed the phytochemical constituents of *Vigna radiata* (L.) Wilczek leaves by qualitative and quantitative methods at three different developmental stages. Phytochemical analysis of these three stages, i.e., young (7 days), mature (20 days), and old (35 days), showed variability in the amount of phytochemicals and antioxidative properties indicating that phytochemical composition and production alters with the growing stages of plants. Qualitative analysis of phytochemicals showed a higher content of metabolites in younger leaves than in mature and old leaves, except for alkaloids, which were found to be higher in mature leaves. Similarly, the quantified value of phytochemicals also matched the qualitative estimation. Furthermore, the antioxidant potential of young, mature and old leaves was also evaluated and found to vary with leaf age. Such studies can provide information on suitable plant stage for consumption or efficient production and extraction of phytochemicals for medicinal and commercial applications.

1. Introduction

Mung beans and their sprouts are rich sources of both stock and bioactive nutrients in many countries. The stock nutrients include carbohydrates and proteins, which constitute essential requirements for energy exchange and primary metabolic activities. Bioactive nutrients are functionally active phytochemicals such as vitamins, phenolics, flavonoids, tannins, saponins, free amino acids, minerals and fibres (Yang *et al*., 2020). These molecules not only scavenge free radicals to alleviate oxidative stress but also modulate certain enzymatic activities, receptors possessing antidiabetic, hyperlipidaemic and anti-inflammatory properties along with defence and other medicinal uses (Ganesan & Xu, 2018). However, functionally active components show differential level of abundance in different parts of the grain, cotyledons, hulls and sprouts. It is also known that high level of bioactive components like total phenolics in mung bean seeds also responsible for high free radical scavenging activity (Yao *et al*., 2012). The hulls of mung bean are evidenced to contain highest concentrations of total phenolics, flavonoids, condensed tannin, saponin, vitexin and isovitexin, than any other parts of the grain (Luo *et al*., 2016). A comparative assessment of functional substances in germinated and non-germinated mung beans has also been reported indicating the germination have positive effect on the production of metabolites (Huang *et al*., 2014). Increased amount of vitamin C, phenolic and flavonoid contents and antioxidant activity have been reported in mung bean sprouts during 9 days germination (Gan *et al*., 2016). There is also a study regarding the variation in phytochemical components during the germination of *Vigna radiata* (L.) Wilczek in two different seasons. The results of this analysis of phytocomponents showed that the biochemical behaviour of germinating seedlings varied in seed lots collected during the summer and rainy seasons (Vijaylaxmi, 2013). It is imperative that the production of bioactive compounds is controlled by several factors such as the site of cultivation, time of cultivation,

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and light and dark periods. Moreover, different parts of the grain and sprout also account for the differential source of bioactive compounds. Despite different existing research involving variation in phytochemical constituents with respect to different parameters, there is scope to determine the effect of growing stages of cotyledon leaves on the availability of phytochemical compounds. The synthesis and availability of different phytochemicals and antioxidant properties in plants are strongly affected by the growing phases. The variation pattern in metabolite profiles during the germination and growth phases is accompanied by dynamic regulation at the transcription level (Tang *et al*., 2014). Because the role of these phytochemical compounds is recently reported in the control of the genetic and epigenetic makeup of the cells during different growth stages transitioning from vegetative and reproductive stages, their availability might change according to the differential expression pattern specific to site or time. The results of different molecular profiling studies stimulated several research projects comprising multiple developmental stages and environmental conditions in different legume crops, including mung beans. Moreover, transcriptomic and metabolomic analyzes of mung bean sprouts have revealed the regulation and nutritional changes during germination (Wang *et al*., 2020). The comprehensive transcriptional patterns of mung bean seedlings after germination remain poorly understood, which may restrict insights into the molecular events triggering metabolism regulation during the transition from vegetative to reproductive phases. Consequently, rationalization awaits further study. Keeping this school of thought in mind, an investigation of different phytochemical components was conducted during different growth phases of first cotyledon leaves after germination to evaluate the changes in the pattern of secondary metabolite production. This study is a primary step toward unraveling the mechanism of the development and commercialization of more qualitatively nutritious food. The present work deals with the preliminary investigation of the methanol extract of *Vigna radiata* to identify major group of phytochemicals that impart medicinal properties to the plant during developing stages. The free radical scavenging activity of these plant leaf extracts is identified using the 2, 2-diphenyl-l-picrylhydrazyl (DPPH) assay, and the hydrogen peroxide scavenging activity was also evaluated.

2. Materials and Methods

The seeds of *Vigna radiata* (L.) Wilczek were collected fromthe commercial market of Malgodown area of Cuttack district of Odisha and authenticated at Department of Botany, Ravenshaw University. The plant parts were examined and identified with the help of regional flora, specimen was further confirmed by refereeing to the book Flora of Odisha available at Department of Botany, Ravenshaw University. The seeds were grown in 36 cell trays during mid-March of summer/spring season and provided optimal sunlight and water for normal growth. Healthy plant leaves were chosen at three different developmental stages – young, mature and old having different growth time duration of 7 days, 20 days and 35 days respectively. Leaves are collected for comparative analysis of active components present among them during the developmental stages.

2.1. Preparation of plant extracts

10 grams of *Vigna radiata* (L.) Wilczek leaves were air dried under shade at room temperature and milled to coarse powder. The obtained dried powder was subjected to successive Soxhlet extraction with (250 ml) methanol for 40 cycles. The extract thus obtained was concentrated to dryness in a flash evaporator under reduced pressure and controlled temperature. The obtained residue was yellowish brown to dark brown colour with thick and sticky paste. The extract was filtered through Whatman No. 1 paper and was quantified before being stored in -4^{o}C refrigerator under airtight condition for further uses.

2.2. Phytochemical Screening Test

The presence of phytochemical constituents in the methanolic extracted young, mature, and old leaves of *Vigna radiata* (L.) Wilczek were carried out to identify metabolites such as alkaloids, total soluble carbohydrate, glycosides, amino acids, tannins, flavonoids, steroids, terpenoids and saponins. For all the qualitative phytochemical screening the final concentration was kept at 1gm of plant leaf extract in one ml of methanol (Agidew, 2022; Kumar *et al*., 2020).

3. Quantitative determination of leaf constituents of *Vigna radiata*

The phytoconstituents particularly, tannins, total phenol content, total flavonoid content and alkaloids found in methanolic extract of *Vigna* leaves during the qualitative screening was quantitatively determined by standard procedures. For all the quantitative measurement the final concentration was kept at 1mg of plant leaf extract in one ml of methanol.

3.1. Determination of tannin content

The tannins were determined by Folin-Ciocalteu method. 1mg/ml of the plant leaf extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent, 1 ml of 35 % Na_2CO_3 solution. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard

Table

Phytochemical Screening	Test protocol	Result
Alkaloids (Wagner's Test)	1 ml of extract treated with 2 ml of Wagner's reagent	Appearance of Reddish-Brown color
Soluble Carbohydrates (Molisch's Solution Test)	1 ml of extract added with few drops of 10% alcoholic solution of α-naphthol, followed by 1ml of concentrated H_2SO_4 along the side of the test tube.	Purple-violet ring at the junction of two liquids confirms the presence of soluble carbohydrates.
Glycosides (Keller-Kiliani Test)	5 ml of extract added with 2 ml of glacial acetic acid, followed by adding one drop of ferric chloride ($FeCl_3$) and 1 ml of concentrated H_2SO_4 into the tube	Appearance of reddish-brown colour
Amino Acids (Ninhydrin Test)	2 ml of extract added with 0.5 ml of Ninhydrin solution and boiled for 2 minutes followed by cooling	Appearance of blue colour
Tannins (Braymer's test)	1ml of extract mixed with 2ml of distilled water along with few drops of 5% ferric chloride solution	Blue-black or blue-green precipitate indicates the presence of tannins
Flavonoids (Aqueous NaOH test)	1 ml of extract dissolved in 3 ml warm distil water and filtered. To this solution added a few drops of 10% aqueous NaOH in 4 ml of solution	A yellow colour appears and become colourless with adding few drops of HCl
Steroids (Liebermann-Burchard)	To 1ml extract added 10 ml of cold acetic acid followed by addition of conc. H_2SO_4 carefully	Colour appears from violet to blue or bluish green
Terpenoids (Salkowski's Test)	In 5 ml of extract added 2 ml of chloroform followed by addition of 3ml of conc. H_2SO_4 to form a layer	Formation of reddish-brown colour layer
Saponins	1 ml extract diluted to 5 ml by adding methanol and incubated at 50°C for 15 minutes. It is followed by cooling and then adding 3ml of distilled water. This solution shaken vigorously for about 5 minutes	Frothing which persisted on warming was taken as evidence for the presence of saponins

solutions of gallic acid (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. Absorbance for the plant leaf extract and standard solutions was measured against the blank at 725 nm with UV/Visible spectrophotometer. The tannin content was expressed in terms of mg of GAE/g (Galvão *et al*., 2018).

3.2. Determination of total phenol content (TPC)

TPC was determined using the spectrophotometric method.1mg/ml of plant leaf extractin triplicate were introduced into test tubes followed by 1.5 ml of Folin-Ciocalteu's reagent (diluted 10 times with water) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were incubated for 30 min and absorption at 765 nm was measured. Total phenolic contents were expressed in gallic acid equivalents (mg per 100-gram dry leaves) (Stanojeviæ *et al*., 2009).

3.3. Total flavonoids content (TFC)

The flavonoids content was determined by aluminium trichloride method using quercetin as reference compound. A volume of 1ml of plant leafextract was added to 0.6 mL of a 5% $NaNO_2$ solution making a total volume of 1.6 ml. The mixture was allowed to stand for 5 minutes and then 1.2 ml of aluminium trichloride (10%) was added and incubated for another 5 min followed by addition of 6mL of NaOH (1M). The final volume of the solution adjusted to 20mL with distilled water. After 15 min of incubation the mixture turned to pink, and the absorbance was measured at 510 nm. The concentration of flavonoid was expressed as mg quercetin equivalent (QE) per gram of extract (Rebaya *et al*., 2015).

3.4. Determination of Alkaloid

The plant leaf extract having concentration of 1mg/ml was oven dried and mixed with 1 ml of dimethylsulphoxide (DMSO) followed by addition of 1ml of 2N HCl and then filtered. This solution was transferred to a separating funnel with the addition of 5 ml of bromocresol green and 5 ml of phosphate buffer (pH 7.4). The mixture was shaken with differential volume of 1, 2, 3 and 4 ml of chloroform by

vigorous shaking. The extract was collected in a 10-ml volumetric flask and diluted to the volume with chloroform in the ratio of 1:1. A set of reference standard solutions of caffeine (20, 40, 60, 80 and 100 µg/ml) were prepared and spectrophotometrically measured. The absorbance for plant leaf extract and standard solutions were determined against the blank at 470 nm. The total alkaloid content was expressed as mg of caffeine per ml of extract (John *et al*., 2014).

4. Antioxidant potential of leaf extract of *Vigna radiata*

In nutraceutical investigations, *in vitro* antioxidant activity assessment methods are often used to screen and confer antioxidant potential to plants or their phytochemicals (Kasote *et al*., 2015). The free radical scavenging potential can vary with the aging or the growing stages of plants. To evaluate this objective, several *in vitro* assays were measured for antioxidant properties of leaves at different age duration were carried out.

4.1. Determination of the radical scavenging ability using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used to determine the free radical scavenging activity of *Vigna radiata* (L.) Wilczek leaf extracts. Briefly, different concentrations (12.5-400µg/ml) of the plant leaf extract of *Vigna* leaves were added with an equal volume of methanolic DPPH solution (0.1mM) and incubated at 37°C for 30 min. The absorbance of the DPPH radical without an antioxidant, i.e., negative control, was also measured. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH Scaveging Effect (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where $A_{o,}$ is the absorbance of the negative control reaction and A_1 is the absorbance in the presence of plant leaf extract.When DPPH reacts with antioxidant, DPPH was reduced, and the colour changed from deep violet to light yellow. This was measured at 517 nm. The experiments were carried out in triplicate and the results expressed as a percentage of the control (Sahu *et al*., 2013).

4.2. Assay of Hydrogen peroxide (H, O,) scavenging activity

Hydrogen peroxide scavenging activity of the plant leaf extract was measured spectrophotometrically. A solution of hydrogen peroxide was prepared with phosphate buffer (pH 7.4) making it to the final concentration of 40mM. Plant leaf extract (100-500µg/ml) were added to 0.6 ml of hydrogen peroxide solution and spectrophotometrically assessed at 230nm. The absorbance was measured after incubating for 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide.

$$\text{Scavanged } H_2O_2 \text{ (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where $A_{o,}$ is absorbance of the negative control reaction, and A_1 is the absorbance inthe presence of the plant leaf extract (Meenatchi *et al*., 2017).

5. Statistical Analysis

All the analyses were performed in triplicate and the results were statistically analyzed and expressed asmean (n=3) ± standard deviation (SD). Statistical calculations were performed in Microsoft office Excel 2010.

6. Results and Discussion

6.1. Qualitative phytochemical analysis

Qualitative analysis for different phytochemical constituents were performed, and it was observed that the methanolic plant leaf extract possesses diverse concentration of metabolites during different growth stages. The 7-day young leaves have more amount of bioactive compounds present than the 20- and 35-days old plant leaf extracts. In a recent study by Wang and associates, the 6-day plant cotyledon leaf extract transcriptome profile reveals upregulation of more than 80 genes that takes part in the regulation of hydroxycinnamic acid and phenyl propanoic acid biosynthesis as well as the synthesis of aromatic amino acid that are precursor of poly phenolic compounds like lignans, coumarins, flavonoids (Wang *et al*., 2020). In another study on the coffee plant and *Moringa oleifera*, the age of the leaf and the kind of solvent do affect the composition of phytochemical profile (Chen *et al*., 2018; Nobossé *et al*., 2018). In table 1, the list of phytochemical constituents presents in the methanolic plant leaf extract indicating majority of the compounds decreases with aging of the leaves except for alkaloids and steroids. These observations can be correlated with the findings of Shukla and Singh, in case of *Papaver somniferum,* more alkaloids are found in the young and mature cotyledonary leaves but with growing stages more alkaloids are in the reproducing organs and trace amount in leaves (Shukla and Singh, 2001). Another recent report regarding the influence of developmental stages on secondary metabolites in medicinal plants by Li and his group states that monoterpenes and sesquiterpenes starts synthesizing in the cotyledon stage but synthesis of specific oils is observed in later stages of growth. In addition, the phytochemical content production also depends on the different vegetative and reproducing stages, environmental

Table 1:

Phytochemical screening of methanolic extract of 3 different developmental stages of *V. radiata* leaves.

Chemical Constituents	7 days extract	20 days extract	35 days extract
Alkaloid	+	+++	++
Carbohydrates	+++	+	+
Glycosides	+	-	-
Phenols	+++	+++	++
Tannins	+++	+	+
Amino acids	+++	++	++
Flavonoids	+++	++	+
Terpenoids	+++	++	+
Saponins	++	-	-
Steroids	++	+	+

+++: Strong positive test; ++: Low positive test; +: Weak positive test; –: Negative test.

stress factors, circadian rhythm, and soil microbial community. On the other hand, glycosides, phenols, flavonoids and saponins were detected in high amount in young leaves and very little to no detection in mature and old leaves (Li *et al*., 2020).

Quantitative determination of the pharmacologically important chemical phytoconstituent indicates the presence of phytochemicals in varying amount during the developmental phases of leaves. The estimation was carried on alkaloids, total phenol content, total flavonoid content, tannins and steroids and interestingly, the qualitative indicators as carried out to screen the phytochemicals correlate with the quantitative estimation of the phytochemicals that were measured using spectrophotometer.

Total alkaloid content reported as the caffeine equivalent were derived from standard curve (y=0.0058x+0.0122, R^2=0.9904). The concentration of total phenolic and tannin contents of 7-, 20- and 35-days leaf extract were determined by the gallic acid equivalent per ml of leaf extract from the standard curve (y=0.0041x-0.0165, R^2=0.9946). Total flavonoid content in the leaf extracts of *Vigna* were calculated against mg quercetin equivalent in the leaf extract of 7, 20 and 35 days with the standard equation of (y=0.0033x+0.0112, R^2=0.998). On the other hand, the steroids concentration in the leaves found to be higher in young leaves (7 days) as compared to older days. Quantitatively, the measured amount of steroid were measured in mg of cholesterol equivalent as calculated from the equation (y=0.0018x-0.0411, R^2=0.9605). The measured values are shown in table 2.

Table 2:

Yield of alkaloids, total phenolics, total flavonoids, tannins, and steroids contents of young, mature, and old leaf extract of *V. radiata.* The value is expressed as mean ± SD from minimum of three independent experiments. TPC and Tannin data was expressed as microgram of gallic acid equivalent (mg GAE) per ml of extract. Steroid data was expressed as microgram of cholesterol equivalent (mg CHO) per ml of extract. Flavonoid data was expressed as microgram of quercetin equivalent (mg QE) per ml of extract. Alkaloid data was expressed as microgram of caffeine equivalent (mg CAF) per ml of extract.

Phytoconstituents	7-days extract	20-days extract	35-days extract
Alkaloids $[\frac{\mu g \text{ of CAF}}{ml \text{ of extract}}]$	34.6 ± 0.529	67.1 ± 1.404	59.5 ±1.7
Total phenolic $[\frac{\mu g \text{ of GAF}}{ml \text{ of extract}}]$	190 ± 4.58	176 ± 4.52	89.2 ± 1.56
Total flavonoids $[\frac{\mu g \text{ of QE}}{ml \text{ of extract}}]$	34.8 ± 3.37	28.5 ± 1.34	15.8 ± 1.34
Tannins $[\frac{\mu g \text{ of GAF}}{ml \text{ of extract}}]$	120.8 ± 4.8	36.9 ± 4.41	37 ± 1.73
Steroids $[\frac{\mu g \text{ of CHO}}{ml \text{ of extract}}]$	66 ± 1.40	46 ± 1	45 ± 0.57

6.3. Antioxidant and free radical scavenging activities of Vigna radiata (L.) Wilczek extract

The DPPH radicals scavenging activity demonstrate the effect of plant leaf extract of different days having antioxidant property through their hydrogen donating ability, which reduces the stable violet DPPH radical to the yellow DPPH. A high percentage of radical scavenging indicating a strong antioxidant activity in the tested sample. The extracts showed concentration dependent antioxidant activity. Furthermore, the extract which contained the considerable amount of total phenolics, and flavonoids effects in reducing DPPH and inhibiting hydrogen peroxide oxidant. The IC_{50} value of scavenging activities on DPPH radical was carried out using an online IC_{50} calculating tool i.e., https://www.aatbio.com/tools/ic 50-calculator (Inc, 2023). The observed values are shown in table 3.

Similarly in case of H_2O_2 scavenging activity, IC_{50} value of scavenging activity of 7-, 20- and 35- days leaves were shown in table 4.

Table 3:

DPPH inhibition percentage and calculated IC_{50} value of *Vigna* leaf extracts of different days.

Concentration (µg/ml)	7-days extract (% Scavenging)	20-days extract (% Scavenging)	35-days extract (% Scavenging)
12.5	46.547	43.544	42.643
25	48.649	46.547	46.246
50	51.351	48.949	48.348
100	52.553	51.652	50.450
200	53.453	52.853	51.952
400	56.156	54.955	52.553
IC_{50}	3.97	5.12	3.96

Table 4:

H_2O_2 scavenging activity and calculated IC50 value of *Vigna* leaf extracts of different days.

Concentration(µg/ml)	7-days extract (% Scavenging)	20-days extract (% Scavenging)	35-days extract (% Scavenging)
100	14.551	13.483	10.787
200	26.854	23.427	16.011
300	38.202	36.966	23.202
400	55.562	54.607	37.579
500	67.416	61.292	44.831
IC_{50}	620.66	330.49	372.45

7. Conclusion

The phytochemical screening in three different stages of leaves - young, mature, and old shown difference in availability of phytochemicals. Qualitative analysis showed that the younger leaves have higher phytochemical content than the older leaves. Quantitative screening indicated that the methanolic plant extract had the highest metabolite content in the young leaf samples, except for alkaloids. Similarly, the DPPH assay and H_2O_2 scavenging activity varied because of the aging process.It is generally accepted that plants undergo series of biochemical and physiological changes during developmental stages viz seedling, vegetative, reproductive and senescence stage. These developmental phases also alter active phytoconstituents and secondary metabolites. Therefore, these changes in the chemical composition of mung beans during germination will also lead to substantial and important changes in their pharmacological properties. These phytoconstituents seem to have the potential to act as a source of useful metabolites and to improve the health status of consumers because of the presence of various compounds that play a vital role towards good health. It also benefits the herbal and phytochemical manufacturing industries to determine the

suitable stage for extraction of plant metabolites for commercial production.

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Bioremediation Potential of *Zinnia elegans* (L.) in Cr enriched Over Burden Soil of Sukinda Chromite Ore Mines, Odisha.

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ABSTRACT

Continues to grow in relation to food safety and sustainable agricultural output. It is a difficult effort to remove Cr from contaminated soils, yet doing so could support agriculture while simultaneously reducing negative environmental effects. Using overburden soil (OBS) of Sukinda, pot investigations were carried out to examine the biochemical and toxicological changes, in an ornamental plant, *Zinnia elegans* (L.). After 30, 45, and 60 days of treatment, the physio-morphological parameters viz total chlorophyll content, proline, protein, reducing sugar content, and antioxidant properties viz. CAT, APX, POX and SOD were examined. Upon T_4 treatment, the plants' length, biomass, chlorophyll and protein contents all decreased. However, the application of a larger percentage of OBS boosted the antioxidative enzymes as well as proline levels, suggesting that the plants' adaptive defense system is related to increasing Cr^{+6} induced oxidative stress. This work shows that non-edible ornamentals can be used to efficiently carry out phytoremediation of Cr^{+6} contaminated soils in mining sectors, stopping the ion's impacts in the food chain.

1. Introduction

A novel green technology known phytoremediation uses plants that accumulate metal or that can withstand metal to clean up soil that has been contaminated with metal. Any environmental factor that keeps plants from realizing their full genetic capacity to carry out metabolic processes under the best growing conditions is often referred to as stress. Trade-offs between vegetative and reproductive growth occur in plant responses to abiotic stress and these trade-offs might vary based on the kind of plant—annual or perennial. Heavy metal phytotoxicity such as Cr^{+6}, disrupts the metabolism of carbohydrates and prevents transpiration and photosynthesis, among other processes. It causes secondary stressors like oxidative stress, dietary stress, lipid peroxidation, hydroxyl radical generation, and H_2O_2, OH- and O_2 accumulation. The plant is therefore experiencing oxidative stress, which is uncontrollable in the absence of antioxidants and impacts the growth and development of the plant (Kramer and Clemens, 2005; Kochian *et al.*, 2005; Lequeux *et al.*, 2010). Employing phytoremediation technology, attempts have been undertaken to lessen the severe contamination caused by harmful hexavalent chromium (Patra *et al.*, 2018a,b,c, 2019, 2020a).

Zinnia elegans L. is an annual blooming plant that is mostly grown for decorative purpose. It can grow in noxious metals and has a rapid growth rate, increased biomass, ease of cultivation, and harvesting (Ehsan *et al.*, 2016). Additionally, using ornamental plants like Zinnia enhances the aesthetic value of the area by improving the air quality in the surrounding area, adding aesthetic value to gardens having the potential to contain alkaloids like anabasin, nor-nicotine, and nicotine. Consequently, *Zinnia elegans* L. is the preferred plant for improved phytoremediation studies when compared to other decorative flowering plants.

Thus, the urge of my current experiment by employing *Zinnia elegans* L. plants are (1) to understand the harmful impacts of hexavalent chromium in soil on biochemical

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changes. (2) Recognize the degree of Cr bioaccumulation. (3) *Zinnia elegans* capacity to hyper-accumulate hexavalent chromium, as well as its absorption and translocation, are assessed by measuring the TI (Tolerance index), Ti (Transportation index) and BCF (Bio-concentration factor).

2. Materials and methods

2.1. Collection of Plant material and Experimental outline

Experiments on pot culture were carried out at Utkal University in Bhubaneswar. *Zinnia elegans* L. dry seeds were taken from RPRC, Bhubaneswar, and grown in department of Botany, Utkal University. Following mercuric chloride of 0.1% (w/v) treatment, the seeds were cleaned with distilled water. In cleansed Petri plates covered in moistened cotton cloths, the treated seeds germinated. The seeds germinated within 4–5 days. In every pot containing varying percentages of Over Burden Soil (OBS) enriched with Cr, (T_0- 100% garden soil as control, T_1- 70% garden soil + 30% OBS, T_2- 50% garden soil + 50% OBS, T_3- 30% garden soil + 70% OBS, and T_4-100% OBS), three seedlings of the same height were planted.

2.2. Biochemical evaluation

The leaf tissues of Zinnia plants that were under treatment and control had a number of biochemical properties analysed.

2.2.1. Photosynthetic pigment

Using cold alkaline acetone and Arnon's (1949) procedures, chlorophyll was extracted. The result was calculated using a formula and given as mg/g fresh weight (FW).

2.2.2. Reducing sugar and Carbohydrates

The carbohydrate content present in the samples was evaluated by the protocol given by Yoshida *et al.*, (1972).

Quantitative determination of reducing sugar is widely followed by the procedure prescribed by Nelson (1944) and Somogyi (1945).

2.2.3. Protein and Proline content

Protein and Proline content were estimated by Lowry *et al.*, (1951) and Bates *et al.*, (1973) methods respectively.

2.3. Antioxidant enzymatic activities

Antioxidant enzymatic properties like superoxide dismutase, peroxidase, ascorbate peroxidase and catalase were measured by Aebi (1984), Marshall and Worsfold (1978), Nakano and Asada (1981) in that order.

2.4. Bioaccumulation and uptake of Chromium

The volume percent of Cr in the shoots and roots of the plants was used to evaluate their capacity for absorption, translocation and phytoextraction. In order to calculate BCF (Bio-concentration factor) and Ti (Transportation index) using the previously used formulas, the metal accumulation in both plants was analysed (Ghosh and Singh, 2005; Zurayk *et al.*, 2002). The methods utilized by Patra *et al.*, (2020b) to assess a plant's ability to grow in the presence of a particular concentration of Cr were applied to determine the tolerance index (TI).

$$\text{BCF} = \frac{\text{Chromium conc. in the plant tissue (mg/ kg)}}{\text{Chromium added in soil (mg/ kg)}}$$

$$\text{Ti} = \frac{\text{Cr conc. of shoot (mg/kg)}}{\text{Cr conc. of root (mg/kg)}} \times 100$$

$$\text{TI} = \frac{\text{Dry wt. of treated plants}}{\text{Dry wt. of control plants}} \times 100$$

3. Results and Discussion

3.1. Impact of Chromium on biochemical properties

It was discovered that the total chlorophyll content increased up to T_2 and then declined as the percentage of Cr-rich OBS increased. Chromium obstructs the synthesis of δ-aminolevulinicacid (ALA) as the initial step in the biosynthesis of tetrapyrrole, which proceeds to the production of heme (Vajpayee *et al.*, 2000). However, Naito *et al.* (1980) proposed that, in addition to regulating ALA synthesis, δ-aminolevulinic acid dehydratase (ALAD) activity may also control the biosynthesis of chlorophyll. When the amount of Cr^{+6} grew in *Zinnia elegans* plants that were 30, 45, and 60 days old, the total sugar content first increased and then gradually dropped (Fig.2). Throughout the growth stage, there were noticeable changes in the decreasing sugar content. Although the sugar level decreases at T_3 and T_4, the plants' reduced sugar content increased across all treatments from 30 days to 60 days. One possible explanation is the disintegration or transformation of other sugar derivatives, including polysaccharides, into non-reducing sugar molecules. On the other hand, the carbohydrate concentrations of *Zinnia elegans* were raised to T_2 and then progressively lowered. Tiwari *et al.* (2009) reported that a substantial drop in the total carbohydrate content occurred as the concentration of Cr increased. In the current in vivo investigation of *Zinnia elegans*, T_2 plants had the highest protein content, followed by T_1. As the amount of hexavalent chromium in the soil increased, the protein content dropped. Because the processes of protein manufacture are disrupted, stress by heavy metal limits the enzymatic

activities containing sulfhydryl group, which also affects protein's normal concentration (Nagoor, 1999). The primary cause behind the decline in protein content is the increased denaturation of proteins brought on by the harmful effects of ROS (Reactive oxygen species) and the conversion of preexisting proteins into amino acids. Hexavalent chromium boosted the proline levels of *Zinnia elegans* plants that were 30, 45, and 60 days old. T_4 has the greatest proline content that was found. Plants that have greater percentages of hexavalent chromium may have accumulated proline as a coping mechanism for the oxidative stress caused by chromium.These outcomes are consistent with our previous research, which looked at *Zinnia elegans* capacity for remediation in Cr^{+6}-contaminated soil and reported that the plants could withstand concentrations of Cr^{+6} of up to 10–50 ppm (Panda *et al.*, 2020).

3.2. *Impact on antioxidant enzymes*

Antioxidant enzymatic properties in 30 days and 60-day-old *Zinnia elegans* were significantly altered by treatment with varying doses of hexavalent chromium rich OBS. As the concentration of hexavalent chromium increased, so did the activities of ascorbate peroxidase (APX), catalase (CAT), peroxidase (GPX) and superoxide dismutase (SOD).

Plants under environmental stress may experience oxidative stress, which produces and activates reactive oxygen species (ROS). Lipid peroxidation is brought on by ROS. Consequently, in order to regulate the amount of Reactive oxygen species and defend the cells from oxidative stress, plants have evolved a defensive mechanism to scavenge the ROS (Vranova *et al.,* 2002).

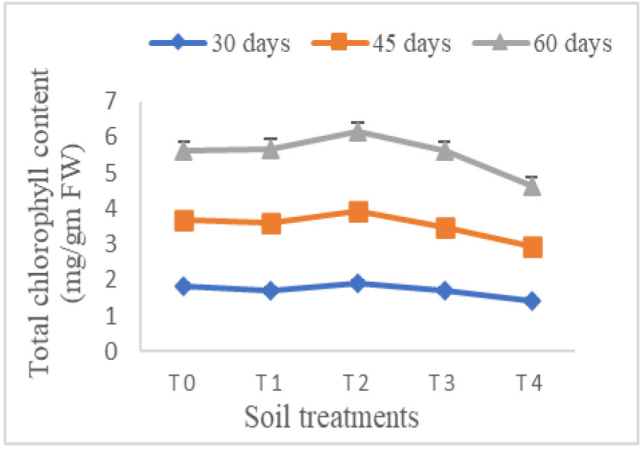

Fig.1. Impact of Cr enriched OBS on total chlorophyll content of 30d, 45d and 60d old *Zinnia elegans* L.

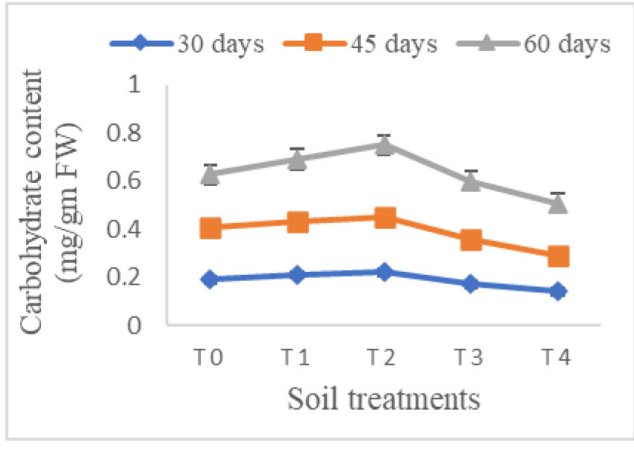

Fig.2. Impact of Cr enriched OBS oncarbohydrate content of 30d, 45d and 60d old *Zinnia elegans* L.

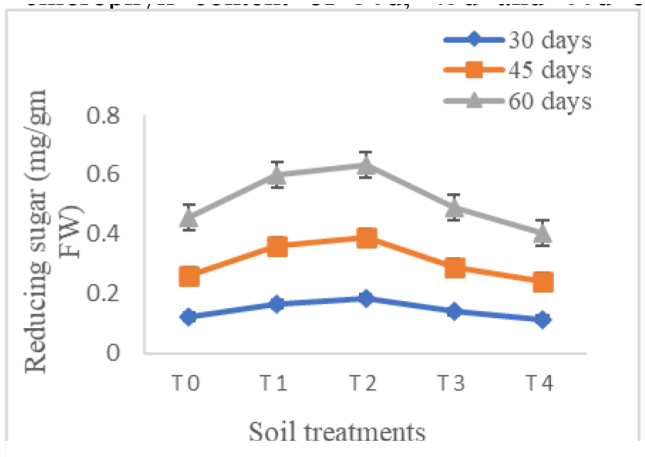

Fig.3. Impact of Cr enriched OBS on reducing sugar content of 30d, 45d and 60d old *Zinnia elegans*L.

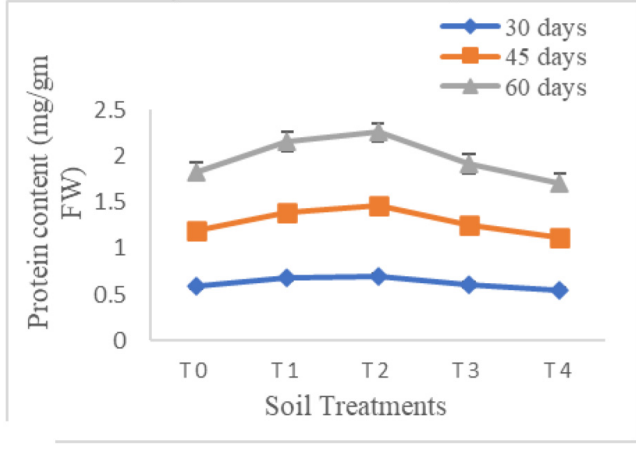

Fig.4. Impact of Cr enriched OBS on protein content of 30d, 45d and 60d old *Zinnia elegans* L.

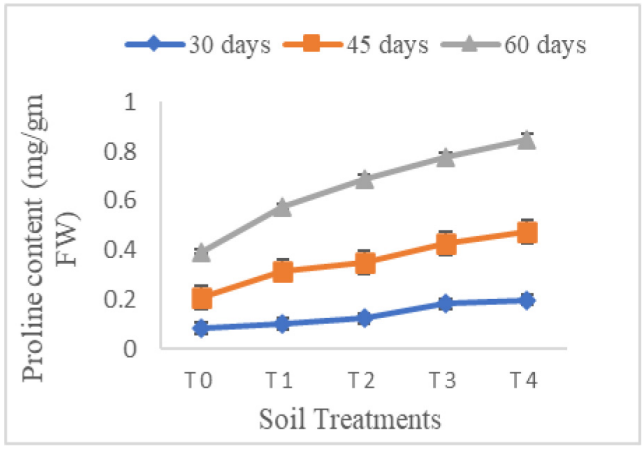

Fig.5. Impact of Cr enriched OBS on proline content of 30d, 45d and 60d old *Zinnia elegans*L.

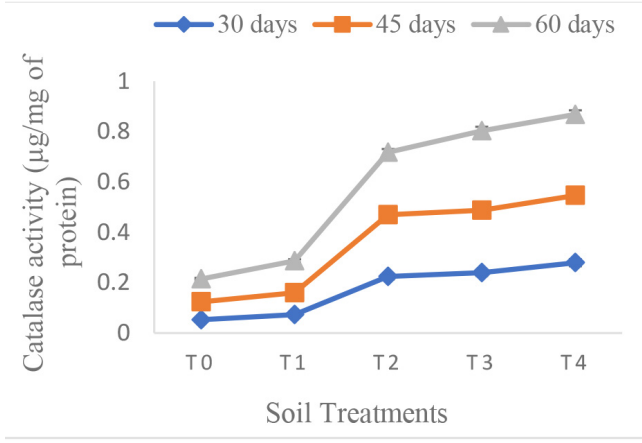

Fig.6. Impact of Cr enriched OBS on catalase activity of 30d, 45d and 60d old *Zinnia elegans*L.

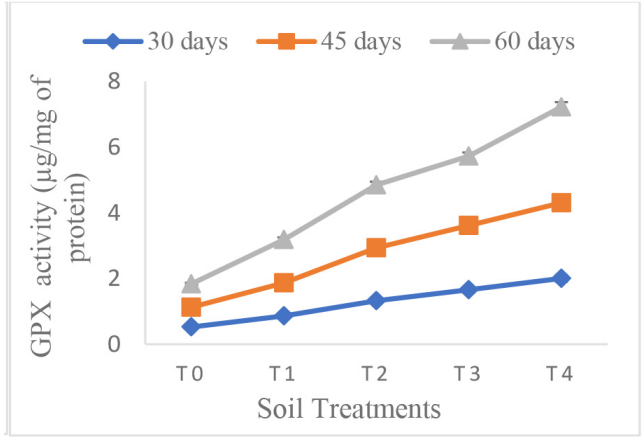

Fig.7. Impact of Cr enriched OBS on peroxidase activity of 30d, 45d and 60d old *Zinnia elegans* L.

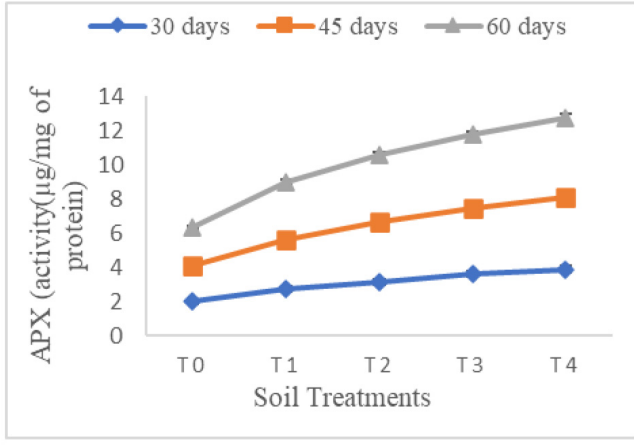

Fig.8. Impact of Cr enriched OBS on APX activity of 30d, 45d and 60d old *Zinnia elegans*L.

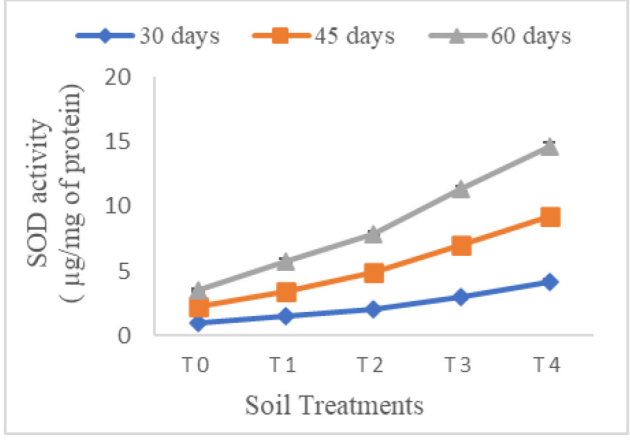

Fig.9. Impact of Cr enriched OBS on SOD activity of 30 d, 45 d and 60 d old *Zinnia elegans* L.

3.3. Bioaccumulation of Chromium

There was a significant difference between the roots and shoots' chromium concentrations. The plants' roots gathered more chromium than their branches. At T_4 treatment, roots showed the highest amount of chromium bioaccumulation. According to Ghosh and Singh (2005), the most prevalent heavy metal tolerance trait is a high Cr bioaccumulation in the roots and less transit to the plant shoots. As the concentration of hexavalent chromium increased from T_1 to T_4, the Ti, TI and BCF all decreased. According to other reports, the plants also generally displayed a tendency for the tolerance index value to decrease as the content of hexavalent chromium increased (Ghosh and Singh, 2005).

Table.1

Effect on bioaccumulation factors of *Zinnia elegans* L.

Soil Treatments	Cr content in shoot (g/kgdry weight)			Cr content in root (g/kgdry weight)			BCF (Bio concentration factor)			TI (Tolerance index)			Ti (Transportation index)		
	30d	45d	60d	30d	45d	60d	30d	45d	60d	30d	45d	60d	30d	45d	60d
T1	7.023± 0.002	7.646± 0.002	8.418± 0.002	8.965± 0.002	9.946± 0.002	11.635± 0.002	3.648	3.869	4.002	164.69	140.84	125.16	65.03	60.87	64.09
T2	9.201 ±0.002	9.601 ±0.002	10.001 ±0.003	13.427 ±0.003	13.988 ±0.003	12.468 ±0.003	2.431	2.462	3.930	155.25	137.93	119.73	57.21	56.85	69.72
T3	9.86 ±0.002	9.826 ±0.002	11.69 ±0.003	17.291 ±0.004	20.812 ±0.005	20.991 ±0.005	0.984	0.992	1.003	119.40	100.73	98.16	35.89	34.27	45.17
T4	10.671 ±0.003	11.32 ±0.003	11.93 ±0.003	22.37 ±0.006	23.705 ±0.007	28.523 ±0.009	0.868	0.986	0.986	67.58	65.14	65.19	34.21	34.89	29.48

4. Conclusion

Chlorophyll, carbohydrate, protein, proline concentration and antioxidant defense system were all impacted by the amount of Cr^{+6} that was present in the soil, demonstrating the plant's capacity to withstand chromium stress. The plants alter a number of metabolic processes to protect themselves against oxidative stress. Furthermore, plants can store Cr in their roots and show a high resistance to Cr in polluted soil. They also develop quickly, have a short life cycle and produce significant biomass. This ornamental plant has little potential of causing heavy metal poisoning to enter the food chain because it is nonedible. Furthermore, *Zinnia* is a beneficial species of plant when used in conjunction with other plants that are necessary to complete the biosystem during mine reclamation once the topsoil at the excavation sites has been restored, such as successional varieties of hardy native perennial grasses and other plants. The current zinnia plant experiment will yield valuable information that may be used to advocate emerging phytoremediation technique for minimizing toxicity of chromium and its practical application in real-world settings.

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Morphobiochemical response of hydroponically grown *Cicer arietinum* L. (*chickpea*) to hexavalent chromium stress and AMF *Claroideoglomus claroideum* inoculation

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ABSTRACT

Chromium (Cr), a heavy metal, is used in industries like electroplating and steel making. The hexavalent (Cr^{+6}) form of Cr is highly toxic to living organisms. The symbiotic association of plant with arbuscular mycorrhizal fungi (AMF) plays a vital role in protecting the plants environmental stress. AMF forms the mutualistic relationship with 80% of vascular plants which has evolved as an adaptive mechanism to enhance the plants' ability to protect themselves from adverse environmental conditions. In the present study, *Cicer arietinum* (chickpea) was grown hydroponically to different concentrations of Cr^{+6} (0, 20, 40 and 60 μM) and without or with AMF *Claroideoglomus claroideum* inoculation. After 30 days of growth, morphological and biochemical parameters were assessed which showed increasing level of Cr^{+6} adversely affected the growth and biochemical parameters of *C. arietinum.* The mycorrhiza-inoculated (M) plants exhibit enhanced growth and higher biomass compared to non-inoculated plants (NM). Biochemical parameters such as photosynthetic pigments, protein, carbohydrate content and catalase activity were higher in M plants than NM plants. However, amino acid and proline content were highest in NM plants compared to M plants. The findings suggest the effectiveness of the *Claroideoglomus claroideum* in mitigating Cr^{+6} stresses in *C. arietinum*, emphasizing the potential of AMF in amelioration of Cr^{+6} stress.

1. Introduction

Food security is important for global development, but contamination of soil with toxic heavy metals, such as Pb, Cd, Cr and Hg is a serious concern (Huang *et al*., 2018). Heavy metals are metallic elements or metalloids with a high atomic density i.e. greater than 4g/cm^3. Rapid industrialization and urbanization introduce these metals into the environment. The increased demand for food due to a growing population leads to the rampant use of fertilizer, agrochemicals, wastewater etc. for agriculture results in contamination of heavy metals into agricultural system.

Chromium (Cr), the 21st most abundant element in earth is a heavy metal found in rocks and water. In nature, Cr exists in two stable forms such as the trivalent (Cr^{+3}) and hexavalent (Cr^{+6}), whereas latter reported to be highly toxic. The hexavalent (Cr^{+6}) form, is especially harmful due to its water solubility (Saha *et al*., 2011). Cr^{+6} contamination comes from both natural and human activities, including industrial processes and mining (Cheng, 2003). Cr^{+6} toxicity also affects the physiological and metabolic processes of plants, leading to changes in stomatal function, decreased water potential, reduced pigment content, disruption of water-mineral linkages, growth inhibition, chlorosis and necrosis (Jena *et al.*, 2016).

To address issues of heavy metal contamination in agricultural system, sustainable farming practices and innovations are necessary. Arbuscular mycorrhiza fungi (AMF) can play a role in mitigating heavy metal toxicity of plants (Hashem *et al*., 2016). Arbuscular mycorrhiza is the symbiotic relationship between fungi and roots of higher plants (Read, 2008). AMF form mutually beneficial

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relationships with plants, enhancing their ability to tolerate stress and preventing metal uptake (Augé, 2001). It also alters the transport of heavy metals within plants, specifically reducing toxicities of aluminum (Al) and manganese (Mn) in acidic soil conditions (Cakmak, 2000). *Cicer arietinum* L. (chickpea) is an important legume crop of the world and is a rich source of vegan protein along with minerals. The present study is designed to study the morpho biochemical response of *C. arietinum* to different levels of Cr^{+6} without and with AMF *Claroideoglomus claroideum* inoculation grown in hydroponics.

2. Materials and methods

2.1 Seed collection and surface sterilization

Seeds of *Cicer arietinum* L. (chickpea) were collected from the Odisha University of Agriculture and Technology (OUAT), Bhubaneswar, Odisha. The collected healthy seeds were surface sterilized using $HgCl_2$ (0.1%) for 5 minutes, followed by washing multiple times with sterile distilled water to remove any residues. Surface sterilized seeds were further subjected to germination.

2.2 Arbuscular mycorrhizal fungi (AMF)

The pure culture of AMF *Claroideoglomus claroideum* (CMCC/AM-2705) was procured from Centre for Mycorrhizal Culture Collection (CMCC), TERI, New Delhi, India. Mass propagation of the starter culture was carried out in pot culture with *Sorghum bicolor* as host plant. Substrate for pot culture constituted of dried soil and sand in the ratio 3:1 (v/v) which was mixed with organic manure at 2:1 ratio (v/v). The Potting mix was cleaned thoroughly by sieving in 2mm sieve and sterilized by autoclaving three times on alternate days at 120° C in 15psi for 30 mins to kill any mycorrhizal spore in the substrate. In a 2 kg poly bag 5 seeds of host plants were sown and after 7 days of seedling growth *C. claroideum* spores were inoculated into the pots. The pots were irrigated with water on alternated days and host plants were allowed to grow for 4 months for AM root colonization and sporulation.

2.3 Preparation of Cr^{+6} treatment solutions

Potassium Dichromate ($K_2Cr_2O_7$) was used as the source of Cr^{+6}. To prepare 100 µM stock solution, 29.419 g of $K_2Cr_2O_7$ was added to 1000 ml of water. Appropriate dilutions were made to the stock solution to get different concentration of Cr^{+6} solutions (20, 40, 60, 80 and 100 µM).

2.4 Seed germination study and determination of LC 50

Ten numbers of surface-sterilized *C. arietinum* (chickpea) seeds were placed over cotton pads saturated with different concentrations of Cr^{+6} (0, 20, 40, 60, 80 and 100 µM) in sterilized petri plates. The seeds on petri plates were kept under dark at 25^{0}C for 5 days for germination. The percentage of germination was recorded and concentration of Cr^{+6} where only 50% of seed germination occurred was considered as LC 50.

2.5 Experimental design

The plant growth experiment was carried out hydroponically in modified Hoagland's solution (Hoagland and Arnon, 1950) after as described by Zeiger (2002). Experiment was randomized with 4 x 2 factorial designs consisting of four Cr^{+6} addition level such as 0 (Control), 20, 40 & 60 µM along with two AMF inoculation treatments such as without AMF inoculation the non-mycorrhizal (NM) and with AMF inoculation the mycorrhizal (M). AMF spore inoculums (Approx. 10 nos. per seed) was given to the overnight soaked *C. arietinum* seed placed on petriplates and after 5 days the germinated seeds were transferred to the hydroponic system for growth with different concentrations of Cr^{+6} (0, 20, 40 & 60 µM) in modified Hoagland's solution. For each treatment 3 replicates were prepared. After 30 days of plant growth different morphological and biochemical parameters were analyzed.

2.6 Growth and morphological parameters

Growth and morphology of *C. arietinum* under different treatments were studied in terms of shoot and root length, shoot and root fresh weight (FW), shoot and root dry weight (DW). The shoot and root length were measured using measuring scale and expressed in cm. The shoot and root fresh weight was determined by weighing in electric balance expressed in grams (g). The fresh biomass was dried in hot air oven at 80° C for 3 hours and dry weight was measured and expressed in g.

2.7 Biochemical parameters

Biochemical parameters such as photosynthetic pigment content (Arnon, 1949), total carbohydrate content by the Anthrone reagent method (Hofreiter, 1962), reducing sugar content (Nelson, 1994), protein content by Lowry method (Lowry *et al.,* 1951), free amino acid (Moore and Stein, 1963) and proline content (Bates *et al*., 1973) in *C. arietinum* shoot were estimated. The antioxidative enzyme catalase (CAT) activity in shoot was estimated following the method of Aebi (1984).

2.9 Estimation of mycorrhizal root colonization

The roots of *C. arietinum* from different treatments were washed thoroughly and were cut into small pieces (1cm), cleared in 10% KOH, bleached in H_2O_2 for 5 min, acidified with 2% HCl and stained in trypan blue (0.05%) as

per Phillips and Hayman (1970). The AMF colonization in each root segment was measured by following the method of Giovannetti and Mosse (1980) which involved gentle squashing of stained root segments on placed glass slide and covered with a cover slip. The squashed root segments were observed under microscope for AMF colonization. The percentage of AMF colonization in root was estimated by following formula:

$$\text{Mycorrhizal colonization (\%)} = \frac{\text{No. of root colonized with AM}}{\text{Total no. of roots inspected}} \text{x } 100$$

2.10 Statistical analysis

The significant difference between parameters by the level of Cr^{+6} addition and AMF inoculation was statistically analyzed by two-way analysis of variance (ANOVA) at $P< 0.05$ using MS excel.

3. Results and discussion

3.1 Germination study

The study of seed germination (%) of *C. arietinum* under different concentrations of Cr^{+6} is presented in Figure 1. At control condition (0 µM) the rate of germination was highest (100%). However, as the concentration of Cr^{+6} increased, there was a decline in the rate of germination. At 80 µM of Cr^{+6}, the seed germination rate was 50%, hence it was considered LC50 where 50% of seeds failed to germinate. The presented data emphasizes the sensitivity of *C. arietinum* seeds to higher levels of Cr^{+6}, indicating the potential toxic impact of Cr^{+6} concentrations on seed germination. Similar findings of Mohanty *et al.* (2015) in *Sesbania sesban* supplemented with different concentrations of Cr^{+6} (5-10000 mg L^{-1}) showed decline in germination of *S. sesban* seeds with increase in Cr^{+6} concentration where LC 50 was at 300 mg L^{-1} Cr^{+6}, which was due to Cr^{+6} toxicity.

3.2 Mycorrhizal root colonization

The AMF root colonization (%) of *C. arietinum* was observed to be decreased with increase in concentration of Cr^{+6} (Figure 2). The highest percentage of root colonization was recorded at Cr^{+6} control (100%) followed by 20, 40 and 60 µM (Cr^{+6}) with 94, 83 and 61% of root colonization respectively. According to the findings of Zhan *et al*. (2017) the root colonization (%) in maize inoculated with AMF *Glomus intraradices* and grown in heavy metal cadmium (Cd) contaminated soil was shown to be maximum under control conditions which was observed to be declined at with increased of levels of Cd (3 and 6 mg/kg soil) suggesting toxicity of heavy metal which can be correlated with present findings.

3.3 Growth and morphological parameters

The plant growth experiment of *C. arietinum* was carried out in hydroponic cups in modified Hoagland's solution with different chromium treatment and AMF inoculation (Figure 3). The various growth parameters studied in the present study like shoot and root length, fresh weight (FW) of shoot and root, dry weight (DW) of shoot and root in both non-mycorrhizal (NM) and mycorrhizal (M) plants of *C. arientinum* showed gradual decrease with increasing Cr^{+6} concentrations (Table 1). The highest values for all parameters were observed at control while the lowest values were recorded at 60 µM. Similar adverse effects of Cr on root and shoot length, as well as biomass yield have been reported in lemongrass grown in soils with chromium contamination (Patra *et al*., 2018; 2019). The report on negative impact of Cr^{+6} on plant growth and development, leading to stunted growth of vegetables crops was also available (Zayed and Terry, 1998). These observations of declining growth and biomass with increasing level Cr^{+6} indicate inverse relationship between Cr^{+6} concentration and decreasing growth in *C. arientinum*.

However, the AMF inoculated plants (M) showed higher growth and biomass than non-inoculated (NM) plants. The findings of Diaz *et al*., (1996) showed increased shoot growth in *Anthyllis cytisoides* inoculated with the arbuscular mycorrhizal fungus (AMF) *Glomus macrocarpum* under Pb and Zn stress respectively. Present results are consistent with Garg and Cheema (2021) where AMF *Claroideoglomus claroideum* effectively mitigated the negative effects of As (V) and As (III) in *C. arientinum* by enhancing root and shoot biomass. The higher growth and biomass in M plants can be positively correlated to mycorrhizal root colonization in M plants of *C. arientinum,* where with increasing concentration of Cr^{+6} there was decline in root colonization. Thus, higher the root colonization, higher the alleviation of Cr^{+6} toxicity as observed in the present study. Hence, present study emphasizes the efficacy of AMF inoculation and root colonization in reducing the adverse effects of Cr^{+6}, enhancing plant resilience under metal stress.

3.4 Biochemical Parameters

3.4.1 Photosynthetic pigment content

The photosynthetic pigments, Chlorophyll a (Chl a), Chlorophyll b (Chl b) and total chlorophyll in both non-mycorrhizal (NM) and mycorrhizal (M) plants showed decreasing trend with increasing Cr^{+6} concentrations (Table 2). The highest values for all parameters were observed at control while the lowest values were recorded at 60 µM. The present study can be correlated with the findings of Mohanty and Patra (2012), where a decline in total chlorophyll

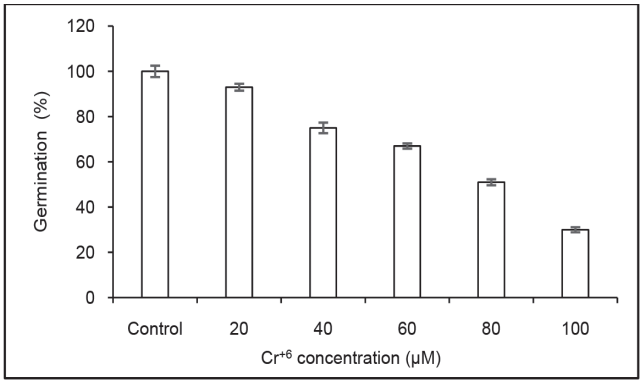

Figure 1. Seed germination under different concentration of Cr^{+6}

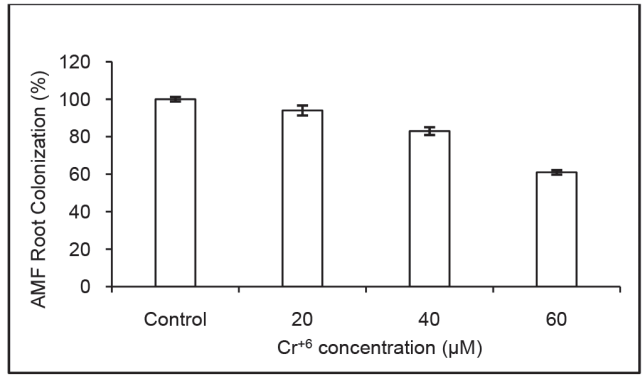

Figure 2. AMF root colonization (%) in *C. arientinum* under different concentration of Cr^{+6}.

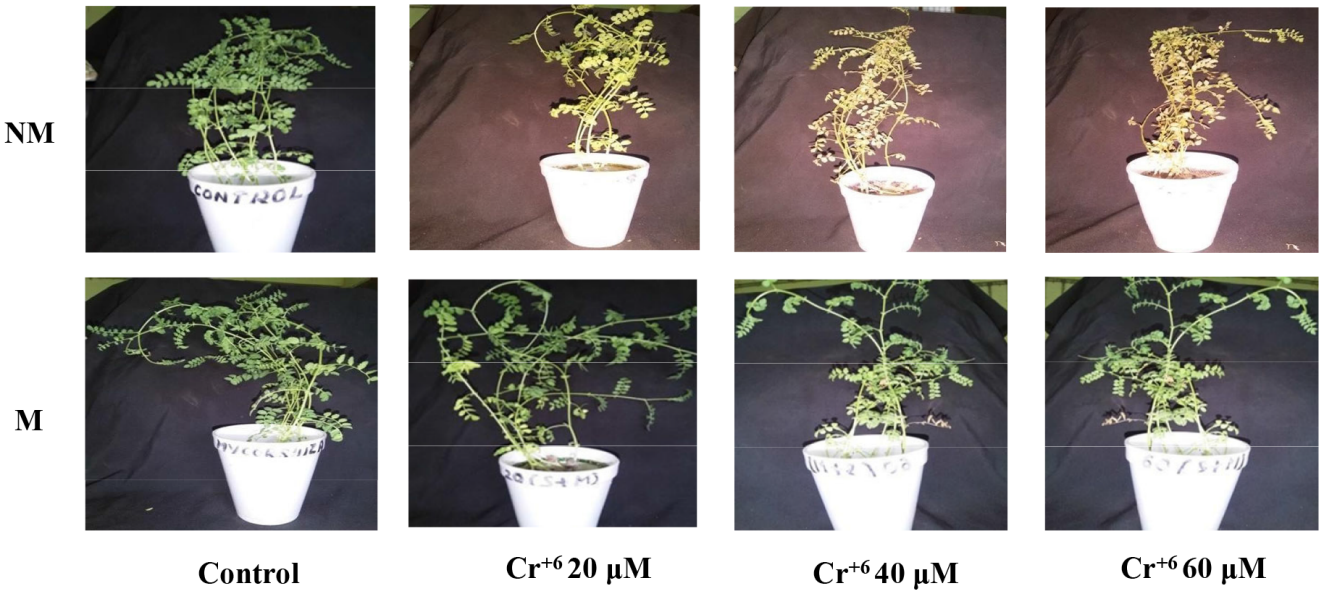

Figure 3: Plant growth of *C. arietinum* in hydroponic cups with modified Hoagland's solution at different concentration of Cr^{+6} in non-mycorrhizal (NM) and mycorrhizal (M) treatments.

Table 1.

Growth parameters of *C. arientinum* under different concentration of Cr^{+6} and AMF treatments.

Cr^{+6} concentration (µM)	Control		20		40		60	
	NM	M	NM	M	NM	M	NM	M
Shoot Length(cm)	20.67±1.20	25.00±1.15	12.13±0.47	19.21±1.67	8.05±0.03	5.09±0.01	7.00±0.29	13.69±0.31
Root Length (cm)	16.66±1.45	19.33±1.45	11.00±1.15	17.33±1.09	6.67±0.33	15.67±1.01	5.17±0.67	9.60±0.31
Shoot FW(g)	1.55±0.02	1.92±0.05	0.74±0.02	0.92±0.02	0.57±0.01	0.83±0.02	0.46±0.01	0.68±0.02
Root FW(g)	0.11±0.01	0.14±0.00	0.06±0.01	0.08±0.04	0.04±0.02	0.05±0.00	0.02±0.001	0.03±0.001
Shoot DW (g)	0.110±0.01	0.183±0.00	0.092±0.001	0.107±0.009	0.073±0.002	0.087±0.002	0.064±0.001	0.079±0.01
Root DW(g)	0.0196 ±0.0001	0.0219 ±0.0001	0.0094 ±0.0002	0.0122 ±0.0002	0.0084 ±0.0002	0.0098 ±0.0002	0.0056 ±0.0001	0.0079 ±0.0001

content in rice and wheat seedlings with increasing Cr^{+6} concentration in hydroponic culture. Wani and Khan (2010) also reported the toxic effects of Cr^{+6} in *C. arietinum* and observed a linear decrease in chlorophyll content with the increase in Cr^{+6} concentration (34, 68 and 136 mg/kg Cr in soil) after a 90-day exposure period.

The M plants showed higher Chl a, Chl b and total chlorophyll content compared to NM plants at different Cr^{+6} treatments. Such observation is similar to the findings of Kullu *et al.*, (2020) in *Brachiaria mutica*, where plants inoculated with *Rhizophagus irregularis* showed increased chlorophyll levels under different concentrations of hexavalent chromium compared to the non-mycorrhizal (NM) one. The study suggests a potential protective effect of mycorrhizal associations in Cr^{+6} stress on plant photosynthetic pigments.

The carotenoid content and chl a/b ratio of *C. arientinum*, in both non-mycorrhizal (NM) and mycorrhizal (M) plants showed enhancement with increasing level of Cr^{+6}. The highest values were observed at 60 µM while the lowest values were recorded at control. However, in M plants the carotenoid content and chl a/b ratio were found to be lower compared to NM plant at all the concentration. The current findings can be compared with the reports of Dhali *et al.,* (2020), which showed an increase in carotenoid content with the increase in concentration (25 to 200 µM) in response to Cr^{+6} in *Macrotyloma uniflorum* and different growth stages (15, 30 and 45 days) in hydroponics. Such findings indicated that a rise in carotenoid content in *C. arientinum* with increasing Cr^{+6} concentrations, suggesting an adaptive photo-protective strategy due to the toxic effects of Cr^{+6} and potential photo-oxidation.

3.4.2 Total carbohydrate, reducing sugar and protein content

The total carbohydrate, reducing sugar and protein content in *C. arientinum* shoots under different Cr^{+6} concentrations for both non-mycorrhizal (NM) and mycorrhizal (M) plants showed declining trend with increasing Cr^{+6} concentrations (Figure 4). The highest value was observed at 0 µM Cr^{+6} while the lowest levels were recorded at 60 µM Cr^{+6}. Raklami *et al*., (2020) reported that in *Medicago sativa*, total sugar content decreased with increasing concentrations of heavy metal Cd and Zn (300 and 600 mg/kg). The present study also correlated with the findings of Patra *et al*., (2020) where *Sesbania sesban* in Cr^{+6} rich environments showed a decrease in protein content with rising of Cr^{+6} which was concentrations, attributed to an increased rate of protein denaturation.

The current study also revealed that M plants showed enhanced total carbohydrate, reducing sugar and protein content compared to NM plants at all Cr^{+6} concentrations. Similar report by Panigrahy *et al*., (2019) in *Eleusine coracana* with *Rhizophagus irregularis* and their findings indicating improved primary metabolite content, supports the idea that AMF inoculation has a positive effect on the nutritional content of plants. Garg and Cheema (2021) reported that the inoculation with different AMF (*Rhizoglomus intraradices*, *Funneliformis mosseae* and *Claroideoglomus claroideum*) in *C.arietinum* has enhanced sugar synthesis under arsenic (As) stress which supports the positive role of AMF symbiosis in plant stress response. Thus, present results suggest that AMF inoculation improves the nutritional status of plants and helps them cope with the toxicity of Cr^{+6} under different concentrations.

3.4.3 Free amino acid and proline content

In both non-mycorrhizal (NM) and mycorrhizal (M) plants, the total free amino acid and proline content in shoot of *C. arientinum* were increased with the increase Cr^{+6} concentrations (Figure 4). The lowest value was observed at control while the highest value was recorded at 60 µM Cr^{+6}. The present results are comparable with the reports of Dhali *et al.,*(2020) where an augmentation in total free amino acid and proline content in *Macrotyloma uniflorum* with rising concentrations of Cr^{+6} (25 to 200 µM) across various growth stages (15, 30 and 45 days) in hydroponic culture. High level of free amino acid indicates protein degradation and as proline is an osmolyte, its concentration increases in plants under stress which maintains the osmotic balance.

The free amino acid and proline content were observed to be lowered in M plants compared to NM plants at all the concentration. Chaturvedi *et al*. (2018) observed elevated proline levels in non-mycorrhizal *Solanum melongena* plants compared to mycorrhizal, grown in Pb and Cd contaminated soils (25 to 100 mg/kg). The study by Ma *et al.,* (2019) reported reduction in proline content in *Helianthus annuus* leaves inoculated with *Claroideoglomus claroideum* under heavy metal stress, indicating potential regulation of osmotic balance and maintenance of cell bioenergetics. Hence, findings of the current study suggest that amelioration of Cr^{+6} toxicity in AMF inoculated plants resulting decrease in proline content of *C. arientinum* compared to non-mycorrhizal plants at different Cr^{+6} treatments.

3.4.4 Anti-oxidative enzyme activity

The present study showed that the anti-oxidative enzyme CAT activity in shoot of *C. arientinum* was

decreased with the increase Cr^{+6} concentrations in both non-mycorrhizal (NM) and mycorrhizal (M) plants (Figure 4). The highest value was observed at control while the lowest value was recorded at 60 µM Cr^{+6}. The present results showed similarity with the reports of Rath and Das (2021) where the anti-oxidative enzyme CAT in hydroponically grown *Vigna mungo* decreased with rising concentrations of Cr^{+6} (100, 150,200, 250 and 300 µM) across various growth stages (15, 30 and 45 days) which lead to ROS mediated oxidative stress in subcellular compartments.

Findings of the present study also revealed that anti-oxidative enzyme CAT activity increased in M plants than NM plant in all the treatments which is similar to the findings of Kullu *et al.* (2020), where the anti-oxidative enzyme activities in *Brachiaria mutica* plants inoculated with *Rhizophagus irregularis* were significantly higher than non-inoculated (NM) plants in all the treatment. Such findings suggest the increase in the rate of scavenging free radicals formed in the plant cell in response Cr^{+6} stress and enhancement of protection against oxidative stress with AMF inoculation resulting in higher stress tolerance potential than the mycorrhiza non-inoculated *C. arientinum.*

3.4.5 Two-way analysis of variance

The two-way analysis of variance (ANOVA) of parameters assessed in *C. arientinum* showed significant variations influenced by different treatments of Cr^{+6} and AMF inoculation. The analysis indicated significant statistical variations between and within the treatments (Table 3).

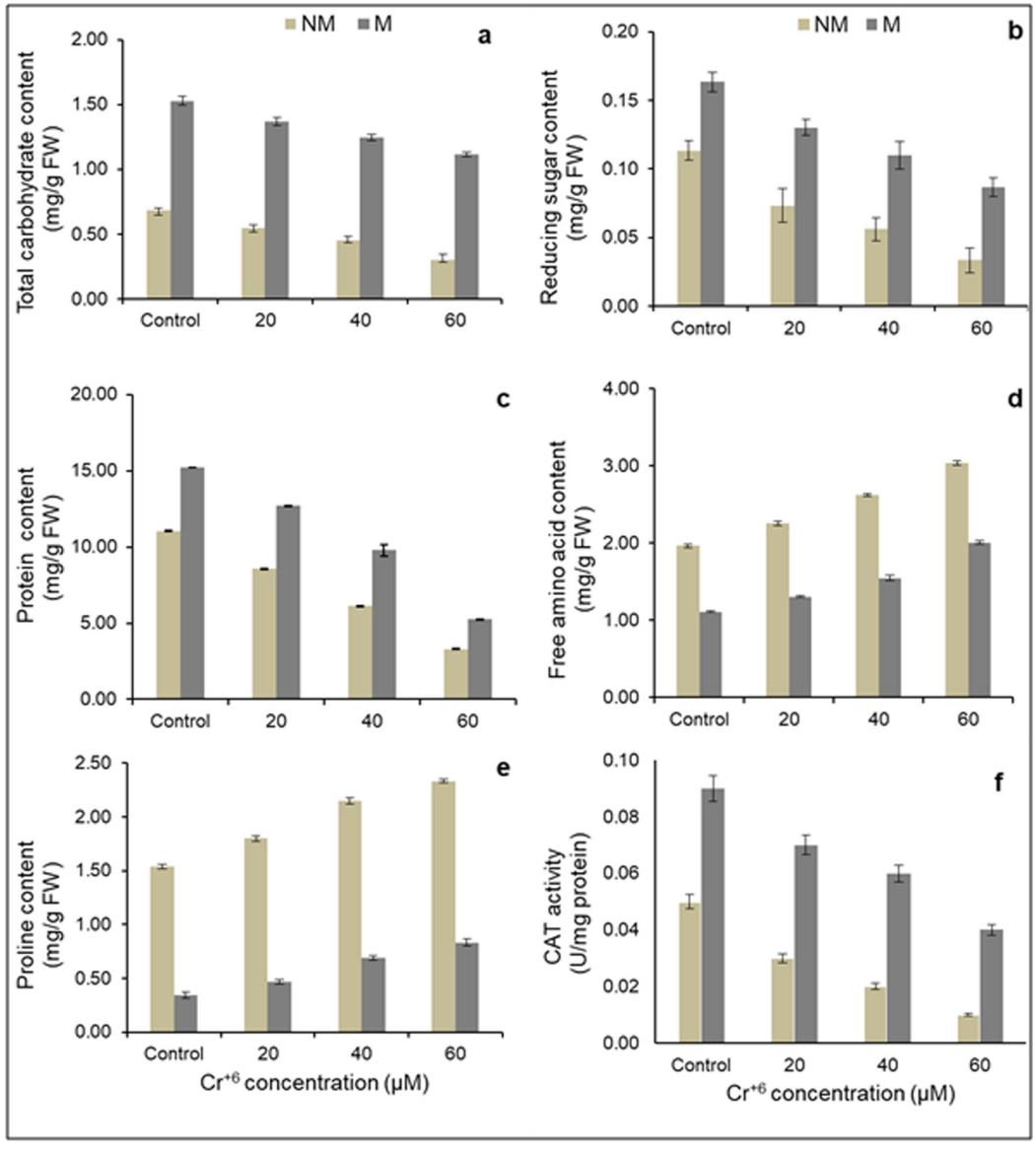

Figure 3. Biochemical parameters a. Total carbohydrate content b. reducing sugar c. protein content d. free amino acid content e. proline content and f. CAT activity in shoot of *C. arientinum* under different concentration of Cr^{+6} and AMF treatments.

Table 3:

F values for two-way ANOVA of different parameters analyzed in *C. arientinum* influneced by various concentrations of Cr^{+6} and AMF treatment.

Parameter	Cr^{+6}	M	Cr^{+6} x M
Shoot length	84.971*	105.758*	1.152
Root length	31.129*	48.266*	2.821
Shoot Fresh weight	947.390*	218.995*	5.706*
Root Fresh weight	335.850*	84.114*	6.393*
Shoot Dry weight	134.518*	103.143*	25.922*
Root Dry weight	3789.655*	487.972*	9.131*
Chlorophyll a	1201.749*	7524.113*	151.537*
Chlorophyll b	335.528*	2695.712*	6.849*
Total Chlorophyll	1659.761*	11622.285*	114.675*
Chlorophyll a:b	62.498*	460.293*	10.817*
Carotenoid	269.867*	190.865*	0.3498
Total carbohydrate	62.701*	1492.761*	0.270
Reducing sugar content	28.070*	73.286*	0.048
Protein content	1773.675*	1485.637*	34.231*
Free amino acid content	590.400*	3136*	8.054*
Proline content	236.792*	5418.632*	13.389*
Catalase activity	12.186*	62.883*	1.674

Asterisk symbol (*) indicate statistically significant *F* value at $p = 0.05$ (n=3),

Cr^{+6}: Cr^{+6} Concentration effect, M: AMF inoculation effect, Cr^{+6} x M: Variable interaction effect

4. Conclusion

In the context of Cr^{+6}stress, AMF *C. claroideum* inoculation exhibits a positive impact on the growth and physiology of *C. arietinum* with enhanced biomass accumulation and favorable alterations in photosynthetic pigments, carbohydrates, proteins and reducing sugar content. The observed augmentation in antioxidative enzyme activities and proline accumulation in mycorrhizal plants suggests a contribution to reactive oxygen species (ROS) scavenging activity. The stress-ameliorative effect of AMF association is more noticeable at lower concentrations of Cr^{+6} (20 µM) compared to higher concentrations (60 µM), exhibiting a positive correlation with the percentage of root association. The findings indicate that AMF association enhances *C. arietinum* tolerance to Cr^{+6} stress, with the effectiveness decline at higher stress levels, possibly inhibiting AMF colonization. Hence, it was concluded that AMF *C. claroideum* inoculation can mitigate low level Cr^{+6} stress in *C. arietinum*.

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A study on isolation, identification and molecular characterization of bacterial pathogens in faecal matter of captive exotic birds in Bhubaneswar smart city

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ABSTRACT

A total of 8 (cloacal and faecal) samples were aseptically collected from different cages of captive exotic birds (Budgerigars, Java sparrow and love birds) from Janpath and Soubhagya nagar area of Bhubaneswar for the isolation, identification and molecular characterization of bacterial flora. All the collected samples were streaked in different selective media for the study of the type of bacteria. Gram staining and biochemical tests were performed for morpho-physiological characterization of the bacteria. This test confirmed the prevalence of *E. coli* among the isolated bacteria. The antibiogram of the isolated bacteria was performed in which isolated bacteria were found to be highly resistant to cloxacillin, tetracycline and ampicillin and highly sensitive to gentamicin and amikacin. Antibacterial properties of extracts from pulp of wood apple and bark of arjun were evaluated in *invitro* condition and are found to be ineffective on the isolated bacteria. The presence of virulence gene *stx1* in the *E. coli* was detected using multiplex PCR. Since, many samples were found to contain *E. Coli,* the handlers must take optimal care during the handling to avoid transmission.

1. Introduction

Now a days, the content of adorning and exotic birds in the urban environment is very common. Birds and their diversity have been considered as good indicators of ecosystem health and in the UK, bird diversity is used as one of the 15 quality of life indicators (Gregory *et al.,* 2003).

The importance of wild birds as probable vectors of disease has resumed empirical interest, especially regarding human health. There are many types of bacterial infections that birds do suffer. Understanding the spread of bacterial pathogens in wild birds may serve as a useful model for examining the spread of other disease organisms, both amongst birds, and from birds to other taxa. Information relating to the normal bacterial flora in gastrointestinal region is limited for the majority of wild bird species, with only few well-studied examples concentrating on bacteria that are zoonotic and relate to avian species of commercial interest (Benskin *et al.,* 2009). Through direct or indirect contact of the diseased or carrier birds many zoonotic diseases are transferred from cageor pet birds to human. Bacteria are one of the most common causes of zoonotic diseases. Hence, proper isolation, identification and characterization of the bacteria collected from infected/carrier birds are essential to control zoonotic diseases.

Wildlife animals that are kept in captivity are very defenceless against opportunistic diseases and they may act as pool of pathogenic bacteria (Ahmed *et al.,* 2007). In most cases, the birds are probably susceptible to these infections due to underlying problems that have allowed for a large bacterial population to overwhelm their normal immunity, or the birds themselves are already weakened due to stress, poor nutrition, or poor husbandry. Most common bacterial pathogens that are noticed in birds includes *Escherichia coli*, *Pasteurella* spp., *Pseudomonas*

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spp., *Salmonella* spp., *Shigella* spp., *Klebsiella* spp. and *Vibrio* spp.

Out of all the bacterial pathogens, *Escherichia coli* can be considered as the most ubiquitous effective enterobacteria in captive animals and is associated with systemic disease in birds (Mattes *et al.,* 2005). The pathogenesis of enteritis by *E. coli* in birds is still not clear, but the presence of diarrheagenic strains may show a public health risk. Although innocuous *E. coli* predominate among the normal flora of the vertebrate intestine, pathogenic forms exist that causedisease of varying severity in humans and otheranimals. Pathogenic strains of *E. coli* are determined by specific virulence factors and their effect in susceptible species. The *Escherichia coli* diarrheagenic (diarrheagenic E. coli - DEC) are an important cause of endemic and epidemic diarrhoea in the world. With the application of polymerase chain reaction (PCR), one can detect genes involved in the pathogenicity of several bacterial isolates, allowing simple identification. In view of this, the small piece of research is focused on isolation, identification and characterization of pathogenic bacteria from faecal samples of captive exotic birds.

2. Material and Methods

2.1. Collection of samples

A total of 8 cloacal swab and faecal samples were collected from 8 different cages of captive exotic birds (Budgerigars, java sparrow and love birds) from Janpath & Soubhagyanagar area of Bhubaneswar.

Each sample was transported to the Mycobacterium Culture Laboratory of Department of Epidemiology & Preventive Medicine, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar-03.

2.2. Isolation of bacterial pathogens

Samples were inoculated in the Nutrient Broth and incubated at 37^0C for 24 hrs. After 24 hours cultureswere streaked on different Enriched media such as EMB, SS, TCBS, KSA and MacC (Himedia, India).

2.3. Preservation of Stock Culture

Bacterial cultures was streaked on the NA slants and preserved at 40 for further use.

2.4. Morphological and Biochemical characterization

The morphology of bacterial isolates was performed with Gram's staining. For biochemical characterization, each isolated bacteria was identified with different tests like oxidase, catalase, urease, citrate, indole, mannitol motility and Triple sugar iron test (Himedia, India). After incubation at 37^0C for 48 hours, the tubes were examined for any change in the slant or butt (Cheesbrough, 1984).

2.5. Haemolytic activity

To determine the haemolytic property of isolated bacteria, the colonies of bacteria were inoculated on Blood Agar media (BA) and incubated at 37^0C for 24 hours. The haemolytic pattern of the bacteria was categorized according to the types of haemolysis produced on BA and this was made as per recommendation of Carter (1986).

2.6. Antibiotic Sensitivity test (ABST)

The drug sensitivity pattern of the isolated bacteria was determined using commercially available antimicrobial discs. In vitro antibiotic sensitivity tests was done using disc diffusion test. Antibiotic discs were placed aseptically on the surface of the inoculated plates with the help of sterile forceps and incubated at 37^0C for 24 hours. After incubation the plates were examined and the diameters of the zone of inhibition were measured using high antibiotic zone scale (Himedia). Depending on the area of the zone diameters for individual antibiotic was recorded as sensitive, intermediate and resistant.

2.7. Anti-bacterial activity of Wood apple and Arjun extract

The following plant materials were collected for the *in vitro* treatment of the bacteria.

Particulars of plant and plant extract collected:

Sl.no.	Local Name	Botanical Name	Source
1	Wood-Apple	*Limonia acidissima*	Ladies Hostel Complex, OUAT
2	Arjuna	*Terminalia arjuna*	Laboratory of the Department of Epidemiology and Preventive Medicine

2.8. Extraction of anti-bacterial compound from Wood apple (Limonia acidissima) and Arjun (Terminalia arjuna):

The fruits (*L. acidissima*) were taken and thoroughly washed under tap water. All the clean samples were separated mechanically into fruit pulp. It was dried in a hot air oven at 60^0 for one week and coarsely powdered using a mixer grinder and stored in an air tight container for further use.

20 gm of dried *L. acidissima* pulp powder was added to 200 ml of methanol and kept in the water bath at 80^0 for 2 hours. The extract was then evaporated using Rotary Vacuum evaporator and then the pure extract was collected in a jar. It was then poured in a glass petri plate and left for drying inside the laminar airflow. Then it was kept in hot air oven at 80^0 till it was dried completely. Then the dried powder was stored in the refrigerator for further use. The powder was mixed with PBS solution at 10mg/ml concentration and mixed thoroughly. The solution was poured into the holes of the cultured plates. Same procedure was repeated for water instead of methanol.

Same procedure of extraction was followed for bark of the Arjun plant.

2.9. Extraction of bacterial genomic DNA

300µL of overnight bacterial culture was taken in 1.5ml tube. 200µL of resuspension buffer was added to it followed by 200µL of lysis buffer. The mixture was incubated at room temperature for 2mins. 20µL of Proteinase K was added to it and was incubated at 65^0C for 20mins with intermediate shake. After incubation, 200µL of precipitation buffer was added to it and then the mixture was transferred to spin column. It was then centrifuged at 10,000rpm for 2mins. 500µL of wash buffer was added to spin column and was centrifuged at 10,000rpm for 2mins. The wash step was repeated for 2 times. Then it was centrifuged at 10,000rpm for 2mins to drag the spin cup. 100µL of elution buffer was added to spin column and was then centrifuged at 10,000rpm for 5mins. It was kept at -20^0C for future use.

2.10. Detection of virulence gene in E. coli

A total of four pairs of primers were used for the detection of virulence associated gene of *E. coli* like *eae* (454bp), *bfpA* (550bp), *stx1* (349bp) and *stx2* (110bp) genes, according to the method described by Costa *et al*., (2010). The reaction mixture for PCR of *E.coli* was prepared by taking ampliqon master mix with forward primer, reverse primer, sample DNA template and nuclease free water and then the amplification was carried out using multiplex PCR. Amplification conditions comprised initial denaturation at 94^0C for 5 min, 35 cycles of 1.5 min at 94^0C, 1.5 min at 50^0 /56^0C and 1.5 min at 72^0C and final extension for 10 min at 72^0C.

2.11. Primers for development of multiplex PCR to detect the pathogenic strains of bacteria

2.12. Agarose gel electrophoresis of PCR product

The PCR products were analysed for positive amplification by agarose gel electrophoresis on 1.5% agarose w/v gels by loading 10µL of PCR product into wells and 100bp DNA ladder was used as a marker. A current of 100V was applied and the PCR products were visualized by UV illumination (BioImaging system).

3. Results and Discussion

Information regarding the normal gastrointestinal bacterial flora is limited for the majority of wild bird species, with the few well-studied examples concentrating on bacteria that are zoonotic or relate to avian species of commercial interest (Benskin *et al*., 2009). However, spreading of bacterial pathogens from one species of birds to another species and from birds to other animals including human beings is considered to be important for study. Hence, the present study tried to focus on presence of pathogenic bacteria in the faecal matter.

Sarker *et al.* (2012) studied the faecal sample of 72 water birds and isolated *E. Coli* (54.16%), *Salmonella spp*

Sl.No.	Genes	Initiators	Sequence (5/- 3/)	Product (bp)
1	*eae*	EAE-1	AAACAGGTGAAACTGTTGCC	
		EAE-2	CTCTGCAGATTAACCTCTGC	454
2	*bfpA*	EP-1	CAATGGTGCTTGCGCTTGCT	
		EP-2	GCCGCTTTATCCAACCTGGT	550
3	*stx1*	STX-1A	CAACACTGGATGATCTAG	
		STX-1B	CCCCCTCAACTGCTAATA	349
4	*stx2*	STX-2A	ATCAGTCGTCACTCACTGGT	
		STX-2B	CTGCTGTCACAGTGACAAA	110

(31.94%), *Staphylococcus spp* (27.78%), *Bacillus spp* (26.38%), and *Proteus* spp. (8.33%) through bacteriological media, biochemical tests and antibiogram profiling. However, literature related to identification of bacterial flora present in the faecal matter of the captive exotic birds is scanty. In the present study, selective culture media like EMB, KSA, SS, TCBS and MacC were used for the identification of bacterial flora. It was found that all the cultures were positive for EMB whereas, with SS four samples were positive and four were negative. Similarly, with KSA four were positive and four were negative. With TCBS all samples were negative. Similarly, five samples were tested positive for lactose fermentation and three samples were negative for lactose fermentation. Through the study, it was noticed that most of the samples were positive for EMB and hence the dominating bacteria was *E. coli.* Subsequently, morpho-physiological characterization was done through biochemical tests and Gram staining from which the domination of *E. coli* in the faecal samples was confirmed. Since the study conducted by Sarker *et al.* (2012) was on water birds, who were more exposed to environmental influence, bacterial flora diversification was more. The present work was on captive/caged exotic birds which restricted the bacterial variety to one or two.

However, no bacteria were found to be involved in rupturing RBCs as confirmed through haemolytic test.

A study conducted by Miranda *et al.* (2008) on the resistivity in poultry intestinal *E. coli* found that the resistance rates of intestinal *E. coli* to all the antimicrobials significantly increased during the course of therapeutic treatment. In the present study antibiotic sensitivity of the isolates were conducted using 16 different antibiotic disks. Out of these, Cloxacillin showed 100% resistance for the isolates, Tetracycline and Ampicillin were found to be resistant for 66.67% of isolates, Amikacin showed sensitivity for 44.45% of the isolates and Cefalexin, Cefotaxime and Gentamicin showed very high sensitivity of 99.99%. It is interesting to note here that another contemporary study conducted in the same laboratory on faecal samples of commercial poultry birds indicated resistance of isolates to all varieties of above mentioned antibiotics. It is likely due to frequent use of antibiotics and consequent developed resistance in poultry birds in the commercial farms.

A report on antimicrobial activity of the extracts of leaves of *Limonia acidissima* against four Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) and five Gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus, Staphylococcus aureus*, *Streptococcus pneumoniae*) was published by Naidu *et al*. (2014). Similar results through bark extract of *Terminalis arjuna* on Gram negative bacteria was reported by Mandal *et al*. (2013). But in the present study, no antibacterial activity of different concentrations of *L. acidissima* and *T. arjuna* extracts was noticed against the *in vitro* cultured isolates. A study conducted by Seeley *et al*. (2014) on a captive flock of budgerigars identified *E. coli* with the virulence gene (*eae*) that contributed to mortality of the birds. Present study detected virulence gene stx1 in *E. coli* at 349 bp using four primers for the genes like *eae, bfpA, stx1* and *stx2* with the help of multiplex PCR.

Table 1

Isolation of bacteria on different selective media

Sl. No.	Sample	EMB (A)	SS (B)	KSA (C)	TCBS (D)	MacC (E)
1	Cage-1	+	-	+	-	LF
2	Cage-2	+	+	-	-	LF
3	Cage-3	+	+	-	-	NLF
4	Cage-4	+	-	+	-	LF
5	Cage-5	+	+	+	-	NLF
6	Cage-6	+	-	-	-	LF
7	Cage-7	+	+	+	-	NLF
8	Cage-8	+	-	-	-	LF

Table 2

Biochemical characterization of final isolates

Sl.	Isolates	Urease	Catalase	Oxidase	Citrate	Indole	Mannitol	Motility	Glucose	Lactose	Sucrose	H_2S	Gas
1	CA1	-	+	-	+	+	+	+	+	+	+	-	+
2	CA3	-	+	-	+	+	+	+	+	+	+	-	+
3	CA4	-	+	-	+	+	+	-	+	+	+	-	+
4	CA5	-	+	-	+	+	+	+	+	+	+	-	+
5	CA6	-	+	-	+	+	+	-	+	+	+	-	+
6	CA7	-	+	+	+	+	+	+	+	+	+	-	+
7	CA8	-	+	-	+	+	+	+	+	+	+	-	+
8	CC1	+	+	-	+	-	+	+	+	-	-	+	+
9	CC2	-	+	-	+	+	+	-	+	+	+	-	+
10	CC4	-	+	-	+	+	+	-	+	+	+	-	+
11	CC5	-	+	-	+	+	+	+	+	+	+	+	+
12	CC7	+	+	-	+	-	+	+	+	-	-	-	+
13	CD5	-	+	-	+	+	+	+	+	+	+	+	+

Table 3

Morphology of isolated bacteria through Gram Staining

Sl. no.	Isolates	Results	Shape of the Bacteria
1	CA1	Gram negative	Small rod
2	CA3	Gram negative	Rod
3	CA4	Gram negative	Rod
4	CA5	Gram negative	Small rod
5	CA6	Gram negative	Small rod
6	CA7	Gram negative	Rod
7	CA8	Gram negative	Rod
8	CC1	Gram negative	Rod
9	CC2	Gram negative	Rod
10	CC4	Gram negative	Rod
11	CC5	Gram negative	Small rod
12	CC7	Gram negative	Small rod
13	CD5	Gram negative	Rod

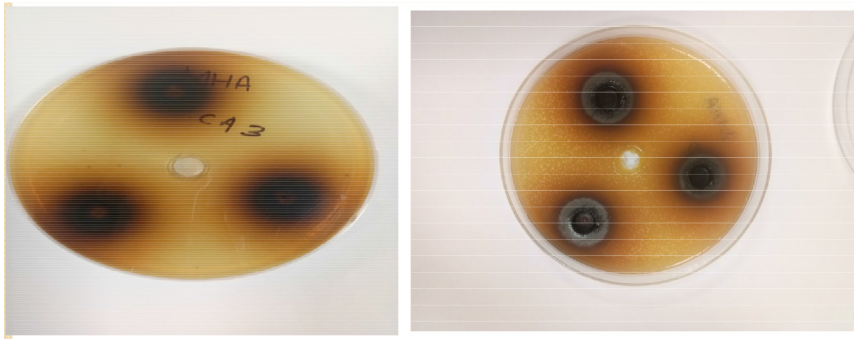
Table 4

Antibiotics sensitivity of the isolated bacteria

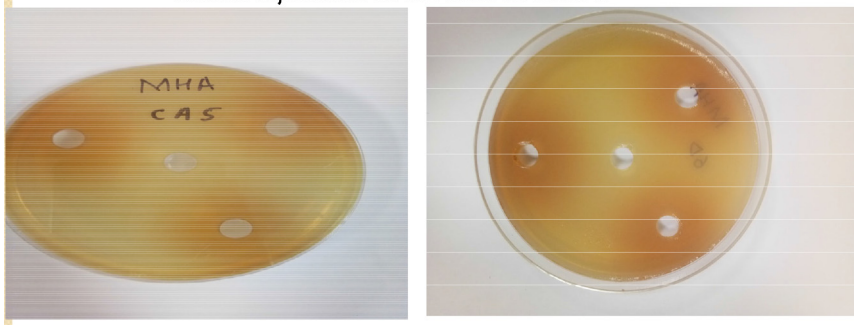
Sl.no.	samples	S=18 R=14 CN	S=22 R=14 CTX	S=20 R=19 AMC	S=15 R=12 GEN	S=21 R=17 CIP	S=11 R=10 CL	S=18 R=13 AZM	S=19 R=14 TE	S=20 R=14 CXM	S=17 R=14 AK	S=19 R=13 AMX	S=17 R=13 AMP	S=18 R=14 CAZ	S=21 R=15 CPZ	S=12 R=11 PB	S=40 R=30 COX
1	CA3	19S	29S	18R	17S	23S	11S	20S	10R	25S	17S	R	13S	11S	19I	11S	R
2	CA6	20S	30S	19R	17S	25S	11S	19S	10R	24S	17S	R	10R	R	21S	13S	R
3	CA8	22S	30S	26S	18S	20I	10R	24S	10R	25S	18S	33S	27S	10R	31S	11R	R
4	CC1	22S	30S	22S	16S	26S	11S	14I	22S	R	16I	R	13R	R	23S	12S	R
5	CC2	22S	31S	10R	18S	16R	11S	25S	R	25S	18S	R	12R	14R	20I	13S	R
6	CC4	22S	27S	22S	17S	26S	12S	18S	10R	24S	16I	R	15I	10R	17I	11R	R
7	CC5	23S	30S	22S	17S	28S	11S	19S	10R	24S	16I	R	13R	R	22S	13S	R
8	CC7	24S	28S	28S	17S	36S	12S	21S	19S	21S	16I	12S	19S	11R	25S	12S	R
9	CD5	21S	28S	21S	17S	28S	11S	18S	10R	24S	17S	R	14I	R	21S	14S	R

R-Resistant, I-Intermediate, S-Sensitive

CN-Cefalexin, CTX-Cefotaxime, AMC-Amoxyclav, GEN-Gentamicin, CIP-Ciprofloxacin, CL-Colistin, AZM- Azithromycin, TE-Tetracycline, CXM-Cefuroxime, AK-Amikacin, AMX-Amoxicillin, AMP-Ampicillin, CAZ-Ceftazidime, CPZ- Cefoperazone, PB-Polymyxin-B, COX-Cloxacillin



Effect of arjuna bark on the cultured bacteria



Effect of wood apple on the cultured bacteria 17

A total of 3 samples were subjected for identification pathogenic strains of *E. coli* through thermal-cycler. The four genes, *eae, bfpA, stx1* and *stx2* were used to identify pathogenic *E.coli,* out of which stx1 gene showed positive result for pathogenic *E. coli* at 349 bp.

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Comparative phytochemical analysis of *Curcuma longa* L. and *Curcuma caesia* Roxb., collected from Kandhamal district of Odisha, India

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ABSTRACT

The present study focuses on comparative phytochemical analysis *Curcuma longa* L. and *Curcuma caesia* Roxb. as both the species have a great medicinal value. The phytochemical analysis of *Curcuma longa* L. revealed presence of alkaloid, glycoside, saponin & carbohydrate and analysis of *Curcuma caesia* Roxb. showed the presence of alkaloid, phenolic compound, flavonoid, saponin, glycoside & carbohydrate. The GC-MS analysis of methanolic extract of *Curcuma longa* L. and *Curcuma caesia* Roxb. was carried out. Bioactive compounds like 9-Tricosene (Z), 1-Undescene & 1-Heptadecene, found in *Curcuma longa* L. have high medicinal properties and bioactive compounds like octanol and oleic acid are the reason behind anti-microbial and anti-inflammatory activity of *Curcuma caesia* Roxb. Functional groups like N-H, O-H, C-≡C, C=O, C=C, C-H, C-F, C-O-C, =C-H, C-O, C≡N, =C-H are present in both turmeric extracts, which can be proved useful in medicinal purpose.

1. Introduction

Turmeric, a plant of family ginger, commonly used as a spice, is also known for its medicinal value. It is the rhizome of the *Curcuma longa* L., belonging to the family Zingiberaceae. For cultivation of *Curcuma longa* L., requires 20^0-30^0 C temperature and a high rainfall (Arulmozhi *et al.,* 2006, Gul *et al.,* 2015, Sasikumar, 2005). This plant is native to South Asian countries and cultivated in India, China, Srilanka, Taiwan, Pakistan, Bangladesh, Thailand and Australia. More than 200 species of Curcuma are found in all over the world out of which 40-50 species are found in India.

Another most valuable species of Curcuma is *Curcuma caesia* Roxb. It is called as Black Turmeric. This species looks like *Curcuma longa* L. but the rhizomes are quite smaller than the rhizomes of *Curcuma longa* L. It is native to Nepal & North-East India, also grown in a few locations in central India. Due to the bluish black hue of its rhizome, it is also known as black turmeric having high economic importance (Devi *et al.,* 2015 and Pandey *et al.,* 2022) reported that bioactive compounds like carotenoids, flavonoids, saponins, tannin, phenolics, terpenoids etc., possess antioxidant, anticancer, anti-asthmatic, anxiolytic, antibacterial, antifungal & antimutagenic properties.

Plants are extremely important for conventional medicine. Turmeric has long been used to treat respiratory infections, wounds, burns, gastrointestinal and liver disorders. The phytochemicals present in plants like alkaloids, flavonoids, terpenoids, steroids, carotenoids play a provital role in pharmaceutical industries. A dimeric derivative of ferulic acid is curcumin. The main bioactive component of turmeric powder is curcumin. Additionally, it

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has choleretic, anti-inflammatory, antiseptic, antibacterial and carminative effects. Now-a-days curcumin is widely used as food additives with coloring, flavoring and preservative properties. Curcumin content of black turmeric is higher than yellow turmeric. Black turmeric contains 14.8% curcumin and yellow turmeric contain 6.3% (Bohra *et al.,* 2021) which can be used not only for scientific research but also in the manufacturing of food and other products.

Pakkirisamy *et al.,* in 2017 conducted the GC-MS, FT-IR, and phytochemical analysis of the methanolic extract of black turmeric *Curcuma caesia* Roxb., and found the presence of tanins, alkaloids, terpenoids, flavonoids, phenol and saponin. Fifteen compounds were found by phytochemicals and chromatography, and functional groups such N-H, O-H, C=C, and CH_3 are also present (Momoh *et al.,* 2022) also in GC-MS analysis of bioactive compounds present in *Curcuma longa* L. rhizome extract (Gaikwad *et al.,* 2022). In view of this, the present study looks at potential phytochemicals found in both turmeric with pharmaceutical importance.

2. Materials and Methods

Collection Site: Odisha's Kandhamal district is famous for its production of natural growing turmeric, which has more healing properties than other turmeric. Kandhamal turmeric earned GI (Geographical Indication) tag from Intellectual property India on 1st April 2019. Kandhamal district is present in the central part of Odisha. This district lies between 19^0-34^0 N to 20^0-36^0 North latitude and 83^0-34^0 East to 84^0-34^0 East longitude.

Collection of Plant Materials: The rhizomes of yellow turmeric (*Curcuma longa* L.) and black turmeric (*Curcuma caesia* Roxb.) were collected from Dharampur, a village of Kandhamal district of Odisha, during the month of December and January because at this time the rhizomes of both turmeric are grown perfectly and cultivated. Then, the rhizomes were packed in paper packets and collected for experimental purpose. The yellow turmeric rhizomes were packed in paper packets having named 'T' and the black turmeric rhizomes were packed in paper packets having name 'BT.' The rhizomes were washed thoroughly so that the soil particles are removed from the rhizomes. Then the extract of both rhizomes was extracted.

Preparation of Methanolic Extract: For the preparation of extract, outer skin of rhizomes was removed and washed thoroughly. Then the rhizomes were chopped into pieces and crushed with mortar pestle. Then they were mixed with 80% methanol. Then the extract of each turmeric was transferred to several volumetric flasks, kept in orbital shakers for one week. Then the extracts of each turmeric were filtered using filter paper. Rotary evaporation was performed on the evaporate all the remaining methanol.

Figure 1 : Showing Rhizomes of *Curcuma caesia* Roxb. & *Curcuma longa* L.

Phytochemical Screening for Turmeric Extract: Two test tubes containing extracts of *Curcuma longa* L. and *Curcuma caesia* Roxb. was taken in 100 ml conical flasks. Then the conical flasks were marked as 'T' for normal turmeric & 'BT' for black turmeric. Then the test for phytochemical screening is carried out.

Test for Alkaloids: After being dissolved in 3 ml of diluted HCl, the extract was filtered out of the mixture. The filtrate underwent the following alkaloid test.

A) DRAGENDROFF'S TEST: A few drops of Dragendroff's reagent were added to the 1ml filtrate. Presence of Alkaloids will be proved by the reddish-brown precipitate.

B) MAYER'S TEST: Two drops of Mayer's reagent were added to 1ml of the filtrate in a test tube. Occurrence of white or creamy precipitate will indicate the test tube as positive.

C) WAGNER'S TEST: To 1ml of extracts in a test tube, two drops of Wagner's reagent were applied. Reddish-brown precipitate will indicate a successful test.

Test for Phenolic Compunds and Tanins:

A) Ferric Chloride test: A few drops of a neutral 5% ferric chloride solution were added after the 1ml of extract had been dissolved in the 5ml of D.W. The presence of phenolic chemical will be indicated by the coloration being dark green.

B) Gelatin's test: 5ml of D.W. and 2ml of a 1% solution of gelatine containing 10% NaCl were added after 1ml of the extract had been dissolved. The presence of phenolic compounds will be revealed by the appearance of white precipitate.

Test for Flavonoids: Alkaline reagent test: 10% ammonium hydroxide solution was used to treat 1 millilitre of the extract. The presence of flavonoids will be confirmed by the formation of yellow fluorescence.

Test for Glycosides: Borntrager's test: In a test tube, 1ml of extract was heated with 1ml of H_2SO_4 for 5 minutes and then filtered while still hot. Following cooling, the filtrate was shaken with an equivalent volume of dichloromethane or chloroform. Dichloromethane or chloroform was divided into layers, and the lowest layer was shaken with half of its volume of diluted ammonia. The ammonial layer will form a rose pink to crimson hue.

Test for Saponin: 3ml of extracts was diluted with D.W & volume was made up to 10 ml. The suspension is shaken vigorously in a graduated cylinder for 15min. Occurrence of a 2cm layer of foam will indicate the presence of saponins.

Test for Carbohydrates:

A) Molisch's test: A few drops of naphthol solution should be added to 1ml of aqueous extract. Shake the test tube and slowly add conc. H_2SO_4 from the side wall. The presence of carbohydrate in the extract will be confirmed by the formation of a red, violet ring at the intersection of two liquids.

B) Fehling's test: 10ml of 50% HCl was mixed into 2ml of the extract in a test tube. Then the mixture was heated in a water both for 30 min. Add 5ml of Fehling's solution & the mixture was boiled for 5 min. Presence of Glycosides will be visible as a brick-red precipitate.

C) Benedict's Test: 2ml of aq. Extract, add few drops of benedict's reagent and heat. Green, yellow, or red colour shows the presence of carbohydrates.

Test for Proteins and Amino Acids:

Biuret's test: In 1.5ml of aq. Extract, add 1.5ml biuret's reagent in test tube for 30 minutes. A violet colour produced will show the present of proteins.

Fourier Transform Infrared Spectrophotometer (Ftir) Analysis: Fourier transform infrared spectrophotometer (FTIR) analysis is the most powerful tools for identifying the types of chemical bonds or functional groups present in extracts. The methanolic extracts of both the turmeric extracts were used for the FTIR analysis.

GC-MS Analysis: The GC-MS test was performed by Central Instrumental Faculty, OUAT, Bhubaneswar. Methanolic extracts were investigated through Gas Chromatography Mass Spectrometry/Mass Spectrometry Electron Ionization (GC-MS/EI) mode. The GC-MS is a Perkin Elmer Clarus 590 model.

3. Result

Phytochemical Screening: The screening for phytochemicals of methanolic extract of both the turmeric was performed (Raaman, 2006).

Test for Alkaloid:

- Dragendroff's test: Reddish brown precipitate was appeared in both test tubes
- Mayer' test: Test tube carrying black turmeric extract showed white or creamy precipitate but in the test tube carrying normal turmeric extract showed slightly less creamy precipitate.
- Wagner's test: Both test tubes showed reddish brown precipitate.

Test for Phenolic Compound:

- Ferric chloride test: Dark green colour was not appeared in both test tubes.
- Gelatin test: Test tube containing black turmeric showed slightly white precipitate but test tube containing normal turmeric did not showed any precipitate.

Test for Flvonoid:

- Alkaline reagent test: Yellow fluorescence is appeared in test tube containing black turmeric extract but not appeared in normal turmeric extract.

Test for Glycoside:

- Borntrager's test: A rose-pink colour is appeared in test tube containing black turmeric extract and light pink colour is appeared in test tube containing normal turmeric extract.

Test for Saponin:

- A 2cm layered foam is appeared in both the test tubes.

Test for Carbohydrates:

- Molisch's test: A red violet ring formed between the junctions of two liquids in both the test tubes.
- Benedict's test: Both the test tubes did not show any green, red, and yellow colour.
- Fehling's test: Test tube containing black turmeric extract showed slightly brick-red precipitate but test tube containing normal turmeric extract did not show any precipitate.

Test for Protiens and Amino Acids:

- Biuret's test: Both the test tubes did not show violet colour.

GC-MS Analysis:

The bioactive substance found in black turmeric and turmeric's methanolic extract is by GC-MS analysis is showed in following tables. Both the turmeric extracts contain very useful bioactive compounds. They can be used as production of various medicines.

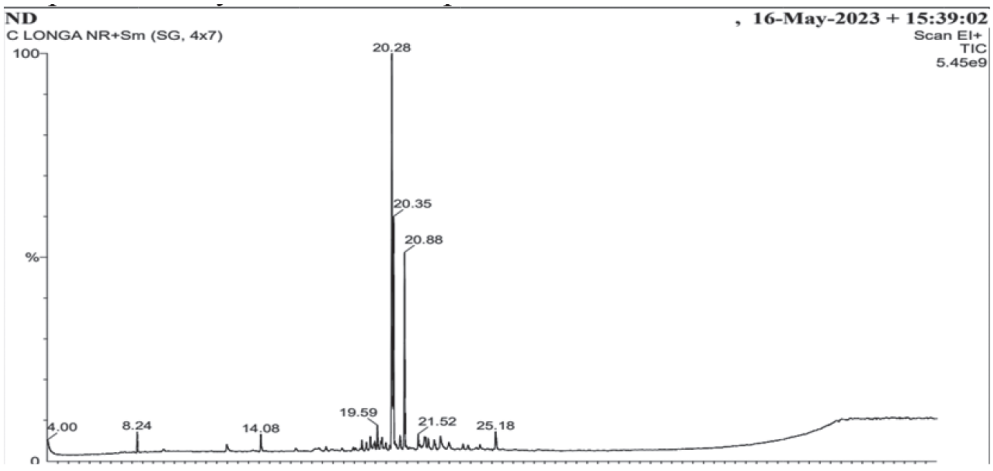

Figure 2: Showing GC-MS Analysis of *Curcuma longa* L.

Table 1:

Bioactive Compounds Found in *Curcuma longa* L.

COMPOUND NAME	MOLECULAR WEIGHT	MOLECULAR FORMULA
5-METHYL-Z-5-DOCOSENE	322	$C_{23}H_{46}$
11-TRICOSENE	322	$C_{23}H_{46}$
9-TRICOSENE, (Z)-	322	$C_{23}H_{46}$
TRIFLUOROACETIC ACID, PENTADECYL ESTER	324	$C_{17}H_{31}F_3O_2$
PENTAFLUOROPROPIONIC ACID, PENTADECYL ESTER	374	$C_{18}H_{31}F_5O_2$
CIS-1-CHLORO-9-OCTADECENE	286	$C_{18}H_{35}Cl$
9-EICOSENE, (E)-	280	$C_{20}H_{40}$
CYCLOUNDECANE, (1-METHYLETHYL)-	196	$C_{11}H_{22}$
5-EICOSENE, (E)-	280	$C_{20}H_{40}$
1-HEPTADECENE	238	$C_{17}H_{34}$
9-NONADECENE	266	$C_{19}H_{38}$
E-15-HEPTADECENAL	252	$C_{17}H_{32}O$
1-UNDECENE, 5-METHYL-	168	$C_{11}H_{22}$
E-14-HEXADECENAL	238	$C_{16}H_{30}O$
1-OCTADECENE	252	$C_{18}H_{36}$
CETENE	224	$C_{16}H_{32}$
1,2-CYCLOHEXANEDIOL, 3-METHYL-6-(1-METHYLETHYL)-, (1.ALPHA.,2.BETA.,3.BET	172	$C_6H_{12}O_2$
9-OCTADECENE, (E)-	252	$C_{10}H_{20}O_2$
ACETIC ACID, CHLORO-, OCTADECYL ESTER	346	$C_{27}H_{45}ClO_3$
1-HENEICOSYL FORMATE	340	$C_{22}H_{44}O_2$

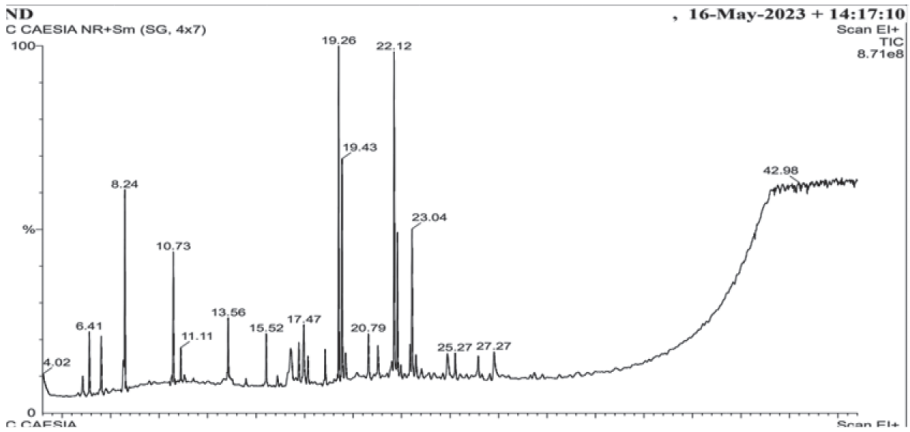

Figure 2 : Showing GC-MS Analysis of *Curcuma caesia* Roxb.

Table 2:

Bioactive Compounds Found in *Curcuma caesia* Roxb.

COMPOUND NAME	MOLECULAR WEIGHT	MOLECULAR FORMULA
2H-PYRAN, 2,2'-[1,10-DECANEDIYLBIS(OXY)] BIS [TETRAHYDRO-	342	$C_{20}H_{38}O_4$
OCTANAL	128	$C_8H_{16}O$
CHLOROMETHYL 2-CHLOROUNDECANOATE	268	$C_{12}H_{22}Cl_2O_2$
Z-8-METHYL-9-TETRADECENOIC ACID	240	$C_{15}H_{28}O_2$
2-N-HEXYLTHIOLANE, S, S-DIOXIDE	204	$C_{10}H_{20}O_2S$
1-DECANOL, 10-[(TETRAHYDRO-2H-PYRAN-2-YL) OXY]-	258	$C_{15}H_{30}O_3$
4-METHYLTHIANE, S, S-DIOXIDE	148	$C_7H_{14}O_2S$
2-ETHYLTHIOLANE, S, S-DIOXIDE	148	$C_6H_{12}O_2S$
2H-PYRAN, TETRAHYDRO-2-(12-PENTADECYNYLOXY)-	308	$C_{20}H_{36}O_2$
2-PIPERIDINONE, N-[4-BROMO-N-BUTYL]-	233	$C_9H_{16}BrNO$
OLEIC ACID	282	$C_{18}H_{34}O_2$
3-N-HEXYLTHIOLANE, S, S-DIOXIDE	204	$C_{10}H_{20}O_2S$
UNDEC-10-YNOIC ACID, 4-METHYL-2-PENTYL ESTER	266	$C_{17}H_{30}O_2$
CYCLOHEXANOL, 2,3-DIMETHYL-	128	$C_8H_{16}O$
BUTYL 9-TETRADECENOATE	282	$C_{18}H_{34}O_2$
CYCLOHEXANOL, 3,3-DIMETHYL-	128	$C_8H_{16}O$
4-METHYL-Z-4-HEXADECEN-1-OL	254	$C_{17}H_{34}O$
BUTYL 9-HEXADECENOATE	310	$C_{20}H_{38}O_2$
4-N-HEXYLTHIANE, S, S-DIOXIDE	218	$C_{11}H_{22}O_2S$
5,5-DIMETHYL-CYCLOHEX-3-EN-1-OL	126	$C_8H_{14}O$

Discussion

The present work aims at novel bioactive compounds from the extracts of rhizomes of indigenous plants *Curcuma longa* L. and *Curcuma caesia* Roxb. Both the turmeric extract and its phytochemical examination revealed that their rhizomes are rich in phytochemicals. The alkaloid tests showed that alkaloid present in both the normal turmeric and black turmeric. The Gelatine test showed the presence of phenolic compounds in black turmeric, which is absent in yellow turmeric. Flavonoid is also present in extract of black turmeric and absent in yellow turmeric. Glycosides present in both the turmeric extracts. Carbohydrate was found to be present in the black turmeric but absent in yellow turmeric. Protein and amino acid test also showed negative response to both turmeric extracts. The presence of highly beneficial bioactive chemicals on the methanolic extracts of *Curcuma longa* L. and *Curcuma caesia* Roxb. was discovered by GC-MS analysis. 9-Tricosene (z) which is found in *Curcuma longa* L. is used as pesticide and 1-Heptadecene is used as making scent and used as cosmetic industries and 1-Undescene, 5-methyl is used for making medicines. The bioactive compounds which are found in *Curcuma caesia* Roxb. are also very useful like Octanol is used in perfume and flavour production and Oleic acid is used as components in many food materials and used as an excipient in pharmaceuticals. Both the turmeric extracts contain useful bioactive compounds which can be used for making various products.

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Genotypic variations of protein banding pattern in indigenous rice genotypes from Koraput, India in relation to drought stress

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ABSTRACT

Indigenous rice landraces are an invaluable resource for restoring genetic diversity. Precise biochemical profiling based on protein banding provides information about the extent of genetic diversity, which helps for effective breeding programs. The present study evaluated SDS-PAGE protein profiling of selected six indigenous rice genotypes from Koraput along with tolerant (N22) and susceptible (IR64) check varieties under control and simulated drought stress. A total of 50 polypeptide bands ranging from 47.7 kDa to 200 kDa were harvested from studied rice genotypes under drought stress, whereas 47 polypeptides bands ranging from 47.13 to 200 kDa was observed in control plants. A total of 17 unique bands were noticed under drought-treated plants, which were absent in control plants. The number of protein bands were higher in some indigenous rice landraces than that of drought-tolerant and susceptible check variety under drought stress. Based on the genetic similarity analysis, some indigenous rice landraces such as Machhakanta, Haladichudi and Kalajeera showed highest genetic similarity with drought tolerant check (N22) variety and formed one cluster. The presence of some unique protein bands in these genotypes indicates their importance for drought tolerance breeding and can be further used for 'omic' studies to better understand stress responses.

1. Introduction

Traditional/indigenous rice genotypes exhibit huge genetic diversity and reservoirs for many potential genes that remain untapped (Samal *et al*., 2018). Different indigenous rice genotypes differ in adaptation to soil types, seeding time, maturity, height, nutritive value, use and other stress-tolerant properties (Arunachalam *et al.*, 2006). These varieties were grown for specific traits like maturity duration, plant stature, panicle features, yield potential, tolerance to biotic and abiotic stresses and other elusive traits like aroma, cooking quality and grain quality (Patra and Dhua, 2003; Roy *et al.*, 2016). The introspection of such diversity is vital for reaching a consensus and making decisions on the conservation and proper utility in breeding programs. Plants respond to drought by regulating gene expression both at the transcriptional and translation levels for the synthesis of stress protein. During water deficit conditions, rice tissue synthesizes more soluble proteins and therefore contributes towards the stress tolerance phenomena (Farooq *et al.*, 2009). Proteins responsible for biosynthesis of osmolytes, ROS scavenging and protection of cellular structure have been identified (Borah *et al.*, 2017). The changes in protein concentration are an indication of stress response towards drought (Qureshi *et al.*, 2007; Choudhary *et al.*, 2009). Majority of the research carried out on this aspect was on high yielding irrigated variety, which is highly susceptible to drought stress. However, detail analysis of protein banding pattern in indigenous rice landraces is lacking.

Koraput district of Odisha, India, is a hotspot of folk rice diversity and secondary centre of origin of Asian cultivated rice (Mishra *et al.*, 2018). Varied agroclimatic

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ecosystems of these region, scarce rainfall, low soil moisture during post-monsoon season and varied topography with an altitude varied from 500 to 1600 mean sea level favour for rice diversity (Mishra *et al.*, 2019). Recently the importance of the region and genetic potentiality of rice diversity in relation to different agronomic traits highlighted (Mishra *et al.*, 2018; Mishra *et al.*, 2019; Panda *et al.*, 2020). However, there is a dearth of biochemical profiling reports and genetic variability studies of protein banding pattern in indigenous rice landraces with respect to drought tolerance. Therefore, the present study aims to characterise the protein banding pattern in selected drought-tolerant folk rice genotypes in relation to drought stress and genotypic relationship will be established between tolerant and susceptible check varieties, which will be helpful for future breeding programs.

Six indigenous rice genotypes namely, Pandakagura, Machhakanta, Haladichudi, Mugudi, Kalajeera and Dangarbayag undar of Koraput, India along with drought-tolerant and susceptible check varieties such as N22 and IR64, respectively were selected for the study. These rice genotypes were popular in the region and were recently identified as most drought-tolerant genotypes of the region (Mishra *et al.*, 2018; Mishra *et al.*, 2019). The rice plants are grown in the hydroponic system using Yoshida nutrient solution and details of growth conditions were recently described in our laboratory by Mishra *et al*. (2018). Briefly, after 30 days of normal growth in a hydroponic system, the plants were treated with drought by application of (36.0%) of polyethylene glycol (PEG)-6000 for 10 days. A control set was also run along with the treatment without the application of PEG. The fresh leaf samples (500 mg) of control and drought-treated plantswere homogenized with extraction buffer (50 mM sodium phosphate buffer (pH 7.0) containing 0.1% polyvinylpyrrolidone (PVP), 0.1% triton X-100 and 10 mM β-Mercaptoehanol). The samples were mixed for 5 minutes in vortex mixer and kept for one hour at room temperature. After incubation, the samples were kept in a boiling water bath for 3 minutes and allowed to cool, and centrifuged at 12000 rpm for 15 min at 4 °C. The crude extract was used for the estimation of protein according to the method of Lowry *et al*. (1951). Protein was denatured by addition of equal volume of SDS-sample buffer and heated at 95 °C for 3 minutes. The Electrophoresis was carried out in vertical electrophoresis unit (Bangalore GeNei maxi.). The 10% Gel cast (20×20 cm and 1mm thickness) was used for the separation of proteins. The protein extract (100 ìg) of different samples was loaded into the wells. Extracted soluble proteins were fractionated by one-dimensional SDS-PAGE gel electrophoresis according to the method of Laemmli (1970). The gel was photographed using gel documentation system (Bio-Rad Gel Doc, California. USA). The number of bands and band density was calculated by the densitometer equipped with the instruments. The presence/absence of bands were transformed into a binary character matrix (1 for presence and 0 for absence of a band at a particular position). The similarity index among different genotypes were constructed by dendogram using protein bands and were measured through Jaccard's similarity coefficient and Nei and Lee Dice coefficient using *PAST-3* (Palaeontological Statistics) software.

2. Results and Discussion

Protein banding pattern in rice genotypes under control and drought stress was shown in Fig. 1. A total of 50 poly peptide bands ranging from 47.7 kDa to 200 kDa were harvested from studied rice genotypes under drought stress, whereas 47 polypeptides bands ranging from 47.13 to 200 kDa was observed in control plants (Table 1). A total of 17 unique bands (66.0 kDa, 66.5 kDa, 72.2 kDa, 75.9 kDa, 80.1 kDa, 85.3 kDa, 92.3 kDa, 93.2 kDa, 93.6 kDa, 98.6 kDa, 99.1 kDa, 110.4 kDa, 116.0 kDa, 129.3 kDa, 130.5 kDa, 136.4 kDa and 154.5 kDa) were noticed under drought treated plants, which were absent in control plants. Similarly, 13 unique bands (67.8 kDa, 73.7 kDa, 77.7 kDa, 78.1 kDa, 78.9 kDa, 109.0 kDa, 109.4 kDa, 119.3 kDa, 123.7 kDa, 124.8 kDa, 135.2 kDa, 162.9 kDa and 170.4 kDa) were present only in control samples. A common protein band of 200 kDa was found in all the tested rice varieties both under control and drought conditions. Under control conditions the protein bands were more or less similar among the genotypes but numbers of protein bands were increased during drought. In this study, the number of protein bands was higher in indigenous rice landraces than that of drought-tolerant and susceptible check variety under drought stress. In particular the drought-tolerant variety (N22) exhibited a greater number of protein bands than that of susceptible (IR64) variety under drought treatment. Some drought responsive polypeptides might be over expressed in the indigenous landraces to withstand the adverse effect of drought as compared to the susceptible variety. It has been additionally observed that different chemical signals transduced under drought stress initiate a variety of genes, prompting the synthesis of proteins and metabolites, providing drought resistance (Mishra *et al.*, 2006). These drought responsive proteins play a vital role for the tolerance mechanism of the studied rice genotypes.

The pair-wise genetic similarity is the measure to categorise the underlying genetic relationship among the genotypes. The genetic similarity was calculated by Jaccard's similarity coefficient and it ranged from 0.012 to 0.667 among the studied rice genotypes under drought stress (Table 2). Based on the genetic similarity analysis, some indigenous rice landraces such as Machhakanta, Haladichudi and

Table 1

List of protein bands with their molecular weights in different rice genotypes under control and drought treatments.

Genotypes	Control			Drought		
	Band No.	Molecular weight (kDa)		Band No.	Molecular weight (kDa)	
Kalajeera	10	1	200.00	9	1	200.00
		2	138.88		2	138.88
		3	114.23		3	114.06
		4	112.57		4	112.08
		5	109.01		5	101.03
		6	100.29		6	99.12
		7	86.62		7	85.36
		8	73.72		8	72.29
		9	63.35		9	63.35
		10	47.14			
Pandakagura	7	1	200.00	11	1	200.00
		2	114.90		2	154.53
		3	113.23		3	115.57
		4	109.49		4	113.73
		5	100.58		5	110.45
		6	79.32		6	102.21
		7	63.35		7	100.58
					8	86.62
					9	75.91
					10	64.92
					11	47.72
Dangarbayagundar	7	1	200.00	8	1	200.00
		2	114.90		2	115.74
		3	102.21		3	111.42
		4	91.40		4	102.66
		5	78.17		5	93.21
		6	64.60		6	79.71
		7	48.42		7	64.60
					8	48.54
Mugudi	10	1	200.00	9	1	200.00
		2	163.00		2	127.06
		3	119.39		3	115.40
		4	114.90		4	103.27
		5	111.10		5	101.77
		6	102.21		6	93.66
		7	89.63		7	80.10
		8	77.79		8	65.88
		9	64.44		9	48.54

		10	48.19			
Macchakanta	12	1	200.00	9	1	200.00
		2	170.41		2	129.34
		3	127.06		3	116.08
		4	115.23		4	103.27
		5	111.75		5	91.40
		6	103.27		6	79.71
		7	101.92		7	66.52
		8	89.63		8	55.93
		9	79.32		9	48.66
		10	65.88			
		11	55.93			
		12	48.78			
Haladichudi	6	1	200.00	7	1	200.00
		2	123.72		2	130.50
		3	103.27		3	103.72
		4	101.77		4	102.07
		5	78.94		5	92.30
		6	67.84		6	80.10
					7	65.72
N22	4	1	200.00	5	1	200.00
		2	124.82		2	136.43
		3	104.02		3	104.94
		4	65.24		4	80.10
					5	66.04
IR64	5	1	200.00	3	1	200.00
		2	135.23		2	98.69
		3	104.79		3	86.20
		4	79.71			
		5	65.56			

Table 2.

Jaccard's similarity coefficient (below diagonal) and Nei and Lee Dice coefficient (above diagonal) among different rice genotypes based on protein banding patterns.

Variety	Kalajera	Pandakagura	Dangarbayagundar	Mugudi	Macchakanta	Haladichudi	N 22	IR 64
Kalajera	1	0.333	0.010	0.011	0.400	0.556	0.500	0.091
Pandakagura	0.500	1	0.125	0.125	0.091	0.200	0.182	0.429
Dangarbayagundar	0.012	0.222	1	0.333	0.100	0.0120	0.010	0.286
Mugudi	0.012	0.222	0.500	1	0.010	0.100	0.010	0.286
Macchakanta	0.571	0.167	0.182	0.012	1	0.400	0.500	0.091
Haladichudi	0.714	0.333	0.012	0.182	0.571	1	0.500	0.010
N 22	0.667	0.308	0.012	0.012	0.667	0.667	1	0.083
IR 64	0.167	0.600	0.444	0.444	0.167	0.013	0.154	1

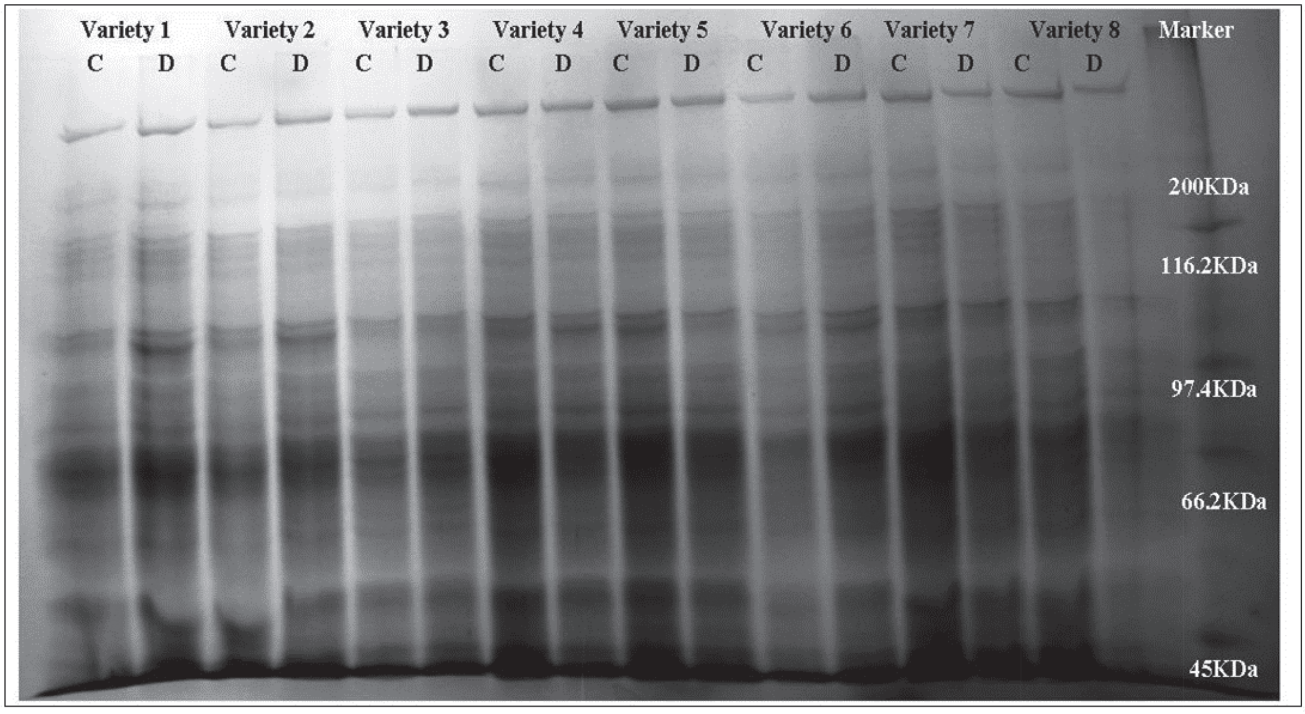

Figure 1: Changes of protein banding pattern in leaf tissue in different folk rice genotypes under control and drought stress. Genotypes 1: Kalajeera; 2: Pandakagura; 3: Dandarbayagundar; 4: Mugudi; 5: Machhakanta; 6: Haladichudi; 7: N22; 8: IR 64.

Kalajeera showed highest genetic similarity with drought tolerant check (N22) variety. Similarly, genetic distance varied from 0.010 to 0.556 among the studied genotypes (Table 2). Based on the results, Machhakanta, Haladichudi, and Kalajeera showed higher Nei and Lee Dice coefficient and showed highly genetically distant from other genotypes.

As genetic diversity is an important requirement for a successful breeding programme and biochemical characteristics such as proteins are powerful tools for the analysis of genetic diversity in rice (Das *et al.*, 2010). Cluster analysis based on the Bray-Curtis paired linkage revealed the percent of similarity in protein banding pattern among rice genotypes presented in Fig. 2. Based on the protein banding pattern under drought stress studied rice genotypes wereforming three major clusters. Indigenous rice landraces such as Machhakanta, Haladichudi, and Kalajeera forming one cluster with drought tolerant check (N22) cultivar showed 66% similarity where as, *Dangarbayagundar* and *mugudi* formed separate cluster with 45% similarity (Fig.2). The genotypes Pandkagura formed separate cluster with drought susceptible check (IR64) cultivar with 55% similarity.

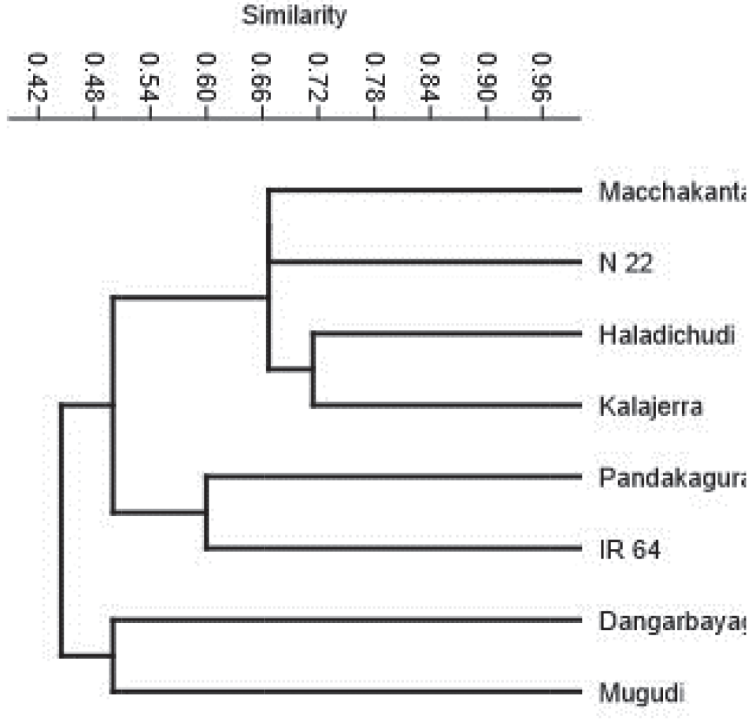

Figure 2: Dendrogram showing the Bray-Curtis similarity index among folk rice genotypes constructed using different protein banding pattern.

3. Conclusion

In conclusion, three genotypes such as Kalajeera, Haldichudi and Machhakanta showed highest genetic similarity with drought-tolerant check variety (N22). Presence of some unique protein bands in these genotypes indicating their importance for varietal identification in drought tolerance breeding. These three indigenous rice genotypes from Koraput are proved to have drought tolerance ability and can later be used for 'omic' studies to better understand stress responses. Further, gene expression studies are aimed to know their molecular mechanism for drought tolerance.

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